11-14-05

AF/1649

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PTO/SB/21 (6-99)

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	09/978,299
Filing Date	October 15, 2001
First Named Inventor	Kevin P. Baker
Group/Art Unit	1649
Examiner Name	Turner, Sharon L.
Total Number of Pages in This Submission: 94	Attorney Docket Number: 39780-2630 P1C3

ENCLOSURES *(check all that apply)*

- [X] **Fee Transmittal Form**
  - [X] **Fee Attached**
- [ ] Amendment / Response
  - [ ] After Final
  - [ ] Version With Markings Showing Changes
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
  - [ ] Copy of Notice
- [ ] Copy of an Assignment
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition Routing Slip (PTO/SB/69) and Accompanying Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, by Assignee to Exclusion of Inventor Under 37 C.F.R. §3.71 With Revocation of Prior Powers
- [ ] Terminal Disclaimer
- [ ] Small Entity Statement
- [ ] Request for Refund
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [X] **Appeal Communication to Group** ***(Appeal Notice, Brief, Reply Brief)***
- [ ] Request for Oral Hearing
- [ ] Status Letter
- [X] **ADDITIONAL ENCLOSURE(S)** ***(PLEASE IDENTIFY BELOW):***
- [X] **EVIDENCE APPENDIX ITEMS 1-7; AND; RETURN POSTCARD**

Remarks

AUTHORIZATION TO CHARGE DEPOSIT ACCOUNT 08-1641 FOR ANY FEES DUE IN CONNECTION WITH THIS PAPER, REFERENCING ATTORNEY'S DOCKET NO. 39780-2630P1C3.

SIGNATURE OF APPLICANT, ATTORNEY OR AGENT

Firm or Individual name	HELLER EHRMAN LLP	BARRIE D. GREENE (Reg. No. 46,740)	
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Signature			
Date	NOVEMBER 10, 2005	Customer Number:	35489

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 on the date indicated below and addressed to: **MAIL STOP APPEAL BRIEF - PATENTS,** Commissioner for Patents, PO Box 1450, Alexandria, Virginia 22313-1450, on this date: **NOVEMBER 10, 2005**

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FEE TRANSMITTAL for FY 2005

Effective 12/08/2004. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 500.00

Complete if Known	
Application Number	09/978,299
Filing Date	October 15, 2001
First Named Inventor	Kevin P. Baker
Examiner Name	Turner, Sharon L.
Art Unit	1649
Attorney Docket No.	39780-2630P1C3

METHOD OF PAYMENT (check one)

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☒ Deposit Account:

Deposit Account Number: 08-1641 (Docket No. 39780-2630P1C3)

Deposit Account Name: Heller Ehrman LLP

The Commissioner is authorized to: *(check all that apply)*

☒ Charge fee(s) indicated below ☒ Credit any overpayments

☒ Charge any additional fee(s) during the pendency of this application

☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Paid
1001	300	2001	150	Utility filing fee	
1002	350	2002	175	Design filing fee	
1003	550	2003	275	Plant filing fee	
1004	790	2004	395	Reissue filing fee	
1005	200	2005	100	Provisional filing fee	

SUBTOTAL (1) ($)

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

			Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		x		=	
Independent Claims		-3** =		x		=	0
Multiple Dependent						=	0

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description
1202	50	2202	25	Claims in excess of 20
1201	200	2201	100	Independent claims in excess of 3
1203	360	2203	180	Multiple dependent claim, if not paid
1204	200	2204	100	**Reissue independent claims over original patent
1205	50	2205	25	**Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Fee Code	Entity Fee ($)	Small Fee Code	Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	120	2251	60	Extension for reply within first month	
1252	450	2252	225	Extension for reply within second month	
1253	1,020	2253	510	Extension for reply within third month	
1254	1,590	2254	795	Extension for reply within fourth month	
1255	2,160	2255	1,080	Extension for reply within fifth month	
1401	500	2401	250	Notice of Appeal	
1402	500	2402	250	Filing a brief in support of an appeal	500.00
1403	1,000	2403	500	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	500	2452	250	Petition to revive - unavoidable	
1453	1,500	2453	750	Petition to revive - unintentional	
1501	1,400	2501	700	Utility issue fee (or reissue)	
1502	800	2502	400	Design issue fee	
1503	1,100	2503	550	Plant issue fee	
1460		1460		Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	790	2809	395	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	790	2810	395	For each additional invention to be examined (37 CFR 1.129(b))	
1801	790	2801	395	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify)

* Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)500.00

SUBMITTED BY

Complete *(if applicable)*

Name *(Print/Type)*	Barrie D. Greene	Registration No. *(Attorney/Agent)*	46,740	*Telephone*	650-324-7000
Signature		*Date*	November 10, 2005	Customer No.	35489

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Examiner: Turner, Sharon L.
Kevin P. BAKER, et al.	)	Art Unit: 1649
Application Serial No. 09/978,299	)	Confirmation No: 4234
Filed: October 15, 2001	)	Attorney's Docket No. 39780-2630 P1C3
For: **PRO195 POLYPEPTIDES**	)	**Customer No. 35489**

EXPRESS MAIL LABEL NO. : EV 582 622 918 US
DATE MAILED: November 10, 2005

ON APPEAL TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

APPELLANTS' BRIEF

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

On April 12, 2005, the Examiner made a Final Rejection to pending Claims 28-35 and 38-40. A Notice of Appeal was filed on September 12, 2005.

Appellants hereby appeal to the Board of Patent Appeals and Interferences from the last decision of the Examiner.

The following constitutes Appellants' Brief on Appeal.

1. REAL PARTY IN INTEREST

The real party in interest is Genentech, Inc., South San Francisco, California, by an assignment of the patent application U.S. Serial No. 09/918,585 recorded July 30, 2001, at Reel 012095 and Frame 0677.

2. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to Appellants, Appellants' legal representative, or Appellants' assignee that will directly affect or be directly affected by or have a bearing on the Board's decision in the present appeal.

3. STATUS OF CLAIMS

Claims 63-65 and 68-70 are in this application.

Claims 1-62 and 66-67 are canceled.

Claims 63-65 and 68-70 stand rejected and Appellants appeal the rejection of these claims.

A copy of the rejected claims involved in the present Appeal is provided in the Claims Appendix.

4. STATUS OF AMENDMENTS

There were no amendments submitted after final rejection. All previous amendments have been entered.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention claimed in the present application is related to an isolated polypeptide comprising the amino acid sequence of the polypeptide of SEQ ID NO:330; the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209722 (Claims 63-65 and 68). The invention is further directed to polypeptides having at least 80%, 85%, 90%, 95%, or 99% amino acid sequence identity to the amino acid sequence of the polypeptide of SEQ ID NO:330; the amino

acid sequence of the polypeptide-of SEQ ID NO:330, lacking its associated signal peptide; or the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209722, wherein the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells (Claims 58-62). The invention is further directed to a chimeric polypeptide comprising one of the above polypeptides fused to a heterologous polypeptide (Claim 69), and to a chimeric polypeptide wherein the heterologous polypeptide is an epitope tag or an Fc region of an immunoglobulin (Claim 70).

The full-length PRO195 polypeptide having the amino acid sequence of SEQ ID NO:330 is described in the specification at, for example, page 23, lines 34-37, page 170, lines 1-7, Example 51, in Figure 132 and in SEQ ID NO:330. The cDNA nucleic acid encoding PRO195 is described in the specification at, for example, Example 51, in Figure 131 and in SEQ ID NO:329. Page 116, lines 23-26 of the specification provides the description for Figures 131 and 132. PRO polypeptide variants having at least about 80% amino acid sequence identity with a full length PRO polypeptide sequence, or a PRO polypeptide sequence lacking the signal peptide are described in the specification at, for example, page 122, lines 34-38, and page 123, lines 2-15. The preparation of chimeric PRO polypeptides, including those wherein the heterologous polypeptide is an epitope tag or an Fc region of an immunoglobulin, is set forth in the specification at page 184, lines 14-35. Examples 100-103 describe the expression of PRO polypeptides in various host cells, including *E. coli*, mammalian cells, yeast and Baculovirus-infected insect cells. Finally, Example 117, in the specification at page 347, line 28, to page 348, line 5, sets forth a adipocyte glucose/FFA uptake assay which shows that PRO195 is an inhibitor of glucose/FFA uptake in adipocyte cells. (see page 348, lines 4-5).

6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. Whether Claims 58-65 and 68-70 satisfy the utility requirement of 35 U.S.C. §101.

II. Whether Claims 58-65 and 68-70 satisfy the enablement requirement of 35 U.S.C. §112, first paragraph.

III. Whether Claims 58-65 and 68-70 satisfy the written description requirement of 35 U.S.C. §112, first paragraph.

7. ARGUMENT

Summary of the Arguments

Issue I: Utility

Patentable utility of the claimed PRO195 polypeptides is based upon the results of the adipocyte glucose/FFA uptake assay for the PRO195 polypeptide. The specification discloses that the adipocyte glucose/FFA uptake assay is designed to determine whether a polypeptide is capable of modulating, either positively or negatively, the uptake of glucose or free fatty acids in adipocyte cells. By making such determinations, the assay identifies polypeptides that are expected to be useful for treating disorders wherein stimulation or inhibition of glucose uptake by adipocytes is expected to be therapeutically effective. Examples of these types of disorders include obesity, diabetes, and hyper- or hypo-insulinemia.

The glucose/FFA uptake assay as described in Example 117 of the instant application was also well known in the art at the time of the effective filing date of the instant application. As demonstrated by the references of record, similar assays were commonly used to identify potential anti-diabetic agents, as well as to study the regulatory mechanisms of important molecules involved in fat cell metabolism, such as leptin.

According to the Examiner, "PRO195 was found to inhibit glucose/free fatty acid uptake. No evidence is offered as to how an inhibitor in such an assay should be used." The Examiner further asserted that "it would be contrary that such inhibitory compounds would be therapeutically beneficial in treating any specific disease. Notably, inhibition of glucose/free fatty acid uptake is contrary to and works in the opposite direction to therapeutics related to insulin resistance and/or diabetes." (Page 6 of the Office Action mailed April 12, 2005).

Appellants respectfully point out that the fact that PRO195 inhibits glucose uptake does not make it useless in such treatment. One of skill in the art would readily understand that a protein which inhibits glucose uptake into adipocytes is a useful therapeutic target, since blocking the function of this protein would decrease the inhibition, and thus increase glucose uptake into adipocytes. One of skill in the art would further understand that antagonists to the PRO195 polypeptide include antibodies, such as the disclosed antibodies which specifically bind the PRO195 polypeptide. PRO195, as an inhibitor of adipocyte glucose uptake, also has utility as a pharmacological tool for investigation of leptin regulation, and associated disorders such as

obesity, in the same way as agents already known and used in the art such as 2-DG, phloretin, and cytocholasin B.

Appellants submit that no further research or investigation is required to show that PRO195 is an inhibitor of glucose uptake, and that its function can be inhibited by molecules such as inhibitory antibodies, the preparation of which is disclosed the specification. In addition, the use of PRO195 in the study of glucose transport and leptin regulation is for the purpose of characterizing not the PRO195 polypeptide itself, but adipocyte glucose transport and the disorders with which it is associated, such as obesity, diabetes, and hyper- and hypo-insulinemia. There is no authority for the proposition that inventions useful in the research setting cannot have patentable utility.

The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration. Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner has made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. Therefore, the legal standard for patentable utility is not absolute certainty. Appellants submit that clear evidence supports the glucose uptake inhibition activity of PRO195. No evidence has been presented to make it more likely than not that one of skill in the art would doubt the truth of this asserted utility of PRO195 as an inhibitor of glucose uptake.

Accordingly, Appellants submit that when the proper legal standard is applied, one should reach the conclusion that the present application discloses at least one patentable utility for the claimed PRO195 polypeptides.

Issue II: Enablement

Claims 58-65 and 68-70 stand rejected under 35 U.S.C. §112, first paragraph, allegedly since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art would not know how to use the claimed invention.

Claims 58-65 and 68-70 further stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking enablement for the claimed polypeptide variants.

Appellants submit that, as discussed above, the PRO195 polypeptides have utility in the treatment of disorders wherein stimulation or inhibition of glucose uptake by adipocytes is expected to be therapeutically effective, such as obesity, diabetes, and hyper- or hypo-insulinemia. Based on such a utility, one of skill in the art would know exactly how to use the claimed polypeptides, without any undue experimentation. Appellants note that the claimed variants, in addition to having at least 80% amino acid sequence identity to SEQ ID NO:330, also have the functional limitation that "the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells." Thus the claimed variants all share the disclosed utility of the PRO195 polypeptide in the treatment of disorders such as obesity, diabetes, and hyper- or hypo-insulinemia. The specification provides ample guidance to allow the skilled artisan to identify those polypeptide variants which meet the limitations of the claims, including a detailed protocol for the adipocyte glucose/FFA uptake assay. The specification also provides detailed guidance as to how to identify and make polypeptides having at least 80% amino acid sequence identity to PRO195 (SEQ ID NO:330). Accordingly, one of ordinary skill in the art would understand how to make and use the recited polypeptide variants without any undue experimentation.

Issue III: Written Description

Claims 58-65 and 68-70 stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking adequate written description for the claimed variant polypeptides.

Current applicable case law holds that biological sequences are not adequately described solely by a description of their desired functional activities. It is, however, well established that a combination of functional and structural features suffices to describe a claimed genus, as discussed in the PTO's own Written Description Guidelines, and as set forth in *Enzo Biochem., Inc. v. Genprobe, Inc.* Appellants note that the claims recite structural features, namely, 80% sequence identity to SEQ ID NO:330, which are common to the genus. The specification provides detailed guidance as to how to identify the recited variants of SEQ ID NO:330, including methods for determining percent identity between two amino acid sequences, as well as listings of exemplary and preferred sequence substitutions. The genus of claimed polypeptides is further defined by having a specific functional activity, namely, that the polypeptide inhibits glucose/FFA uptake by adipocytes. Example 117 of the present application provides step-by-step guidelines and protocols for the adipocyte glucose/FFA uptake assay assay. By following the

disclosure in the specification, one skilled in the art can easily test whether a gene encoding a variant PRO195 protein inhibits glucose/FFA uptake by adipocytes.

Accordingly, a description of the claimed genus has been achieved by the recitation of both structural and functional characteristics.

These arguments are all discussed in further detail below under the appropriate headings.

ISSUE I: Claims 58-65 and 68-70 satisfy the utility requirement of 35 USC §101

Claims 58-65 and 68-70 stand rejected under 35 U.S.C. §101 because allegedly "the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility." (Page 3 of the Office Action mailed April 12, 2005).

Appellants submit, for the reasons set forth below, that the specification discloses at least one credible, substantial and specific asserted utility for the claimed PRO195 polypeptides.

A. The Legal Standard for Utility

According to 35 U.S.C. § 101:

> Whoever invents or discovers any new and *useful* process, machine, manufacture, or composition of matter, or any new and *useful* improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title. (Emphasis added).

In interpreting the utility requirement, in *Brenner v. Manson,*[1] the Supreme Court held that the *quid pro quo* contemplated by the U.S. Constitution between the public interest and the interest of the inventors required that a patent applicant disclose a "substantial utility" for his or her invention, i.e. a utility "where specific benefit exists in currently available form."[2] The Court concluded that "a patent is not a hunting license. It is not a reward for the search, but compensation for its successful conclusion. A patent system must be related to the world of commerce rather than the realm of philosophy."[3]

Later, in *Nelson v. Bowler,*[4] the C.C.P.A. acknowledged that tests evidencing pharmacological activity of a compound may establish practical utility, even though they may not

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[1] *Brenner v. Manson,* 383 U.S. 519, 148 U.S.P.Q. (BNA) 689 (1966).

[2] *Id.* at 534, 148 U.S.P.Q. (BNA) at 695.

[3] *Id.* at 536, 148 U.S.P.Q. (BNA) at 696.

[4] *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. (BNA) 881 (C.C.P.A. 1980).

establish a specific therapeutic use. The court held that "since it is crucial to provide researchers with an incentive to disclose pharmaceutical activities in as many compounds as possible, we conclude adequate proof of any such activity constitutes a showing of practical utility."[5]

In *Cross v. Iizuka,*[6] the C.A.F.C. reaffirmed *Nelson*, and added that *in vitro* results might be sufficient to support practical utility, explaining that "*in vitro* testing, in general, is relatively less complex, less time consuming, and less expensive than *in vivo* testing. Moreover, *in vitro* results with the particular pharmacological activity are generally predictive of *in vivo* test results, i.e. there is a reasonable correlation there between."[7] The court perceived "No insurmountable difficulty" in finding that, under appropriate circumstances, "in vitro testing, may establish a practical utility."[8]

Furthermore, M.P.E.P. §2107.03 (III) states that:

> "If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process."

Thus, the legal standard accepts that *in vitro* or animal model data is acceptable utility as long as the data is "reasonably correlated" to the pharmacological utility described. The case law has also clearly established that Appellants' statements of utility are usually sufficient, unless such statement of utility is unbelievable on its face.[9] The PTO has the initial burden to prove that Appellants' claims of usefulness are not believable on their face.[10] In general, an Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope."[11,12]

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[5] *Id.* at 856, 206 U.S.P.Q. (BNA) at 883.

[6] *Cross v. Iizuka*, 753 F.2d 1047, 224 U.S.P.Q. (BNA) 739 (Fed. Cir. 1985).

[7] *Id.* at 1050, 224 U.S.P.Q. (BNA) at 747.

[8] *Id.*

[9] *In re Gazave*, 379 F.2d 973, 154 U.S.P.Q. (BNA) 92 (C.C.P.A. 1967).

[10] *Ibid.*

[11] *In re Langer*, 503 F.2d 1380,1391, 183 U.S.P.Q. (BNA) 288, 297 (C.C.P.A. 1974).

Compliance with 35 U.S.C. §101 is a question of fact.[13] The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the totality of the evidence under consideration.[14] Thus, to overcome the presumption of truth that an assertion of utility by the applicant enjoys, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence.

The well established case law is clearly reflected in the Utility Examination Guidelines ("Utility Guidelines")[15], which acknowledge that an invention complies with the utility requirement of 35 U.S.C. §101, if it has at least one asserted "specific, substantial, and credible utility" or a "well-established utility." Under the Utility Guidelines, a utility is "specific" when it is particular to the subject matter claimed. For example, it is generally not enough to state that a nucleic acid is useful as a diagnostic without also identifying the conditions that are to be diagnosed.

In explaining the "substantial utility" standard, M.P.E.P. §2107.01 cautions, however, that Office personnel must be careful not to interpret the phrase "immediate benefit to the public" or similar formulations used in certain court decisions to mean that products or services based on the claimed invention must be "currently available" to the public in order to satisfy the utility requirement. "Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility."[16] Indeed, the Guidelines for Examination of Applications for Compliance With the Utility Requirement,[17] gives the following instruction to patent examiners:

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[12] *See also In re Jolles*, 628 F.2d 1322, 206 U.S.P.Q. 885 (C.C.P.A. 1980); *In re Irons*, 340 F.2d 974, 144 U.S.P.Q. 351 (1965); *In re Sichert*, 566 F.2d 1154, 1159, 196 U.S.P.Q. 209, 212-13 (C.C.P.A. 1977).

[13] *Raytheon v. Roper*, 724 F.2d 951, 956, 220 U.S.P.Q. (BNA) 592, 596 (Fed. Cir. 1983) *cert. denied*, 469 US 835 (1984).

[14] *In re Oetiker*, 977 F.2d 1443, 1445, 24 U.S.P.Q.2d (BNA) 1443, 1444 (Fed. Cir. 1992).

[15] 66 Fed. Reg. 1092 (2001).

[16] M.P.E.P. §2107.01.

[17] M.P.E.P. §2107 II(B)(1).

"If the applicant has asserted that the claimed invention is useful for any particular practical purpose . . . and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility."

B. The Data and Documentary Evidence Supporting a Patentable Utility

Appellants respectfully submit that Appellants rely on the results of the adipocyte glucose/FFA uptake assay for patentable utility of the PRO195 polypeptides, and that the adipocyte glucose/FFA uptake assay data for the PRO195 polypeptide is clearly disclosed in the instant specification under Example 117.

The adipocyte glucose/FFA uptake assay is designed to determine whether a polypeptide is capable of modulating, either positively or negatively, the uptake of glucose or free fatty acids in adipocyte cells. By making such determinations, the assay identifies polypeptides that are expected to be useful for treating disorders wherein stimulation or inhibition of glucose uptake by adipocytes is expected to be therapeutically effective. Examples of these types of disorders include obesity, diabetes, and hyper- or hypo-insulinemia.

The adipocyte glucose/FFA assay is performed as follows: primary rat adipocyte cells are plated on a 96 well plate and incubated overnight with media supplemented with PRO195 polypeptide. After the initial overnight incubation, samples of the media are taken at hour 4 and hour 16 and residual glycerol, glucose and FFA are measured. After the hour 16 sample is taken, insulin is added to the media and the adipocytes are allowed to incubate for an additional 4 hours. After this final 4 hour incubation, another sample is taken and residual glycerol, glucose and FFA is measured again. As a control, identical incubations and samplings are performed on cells that have been incubated overnight in media initially supplemented with insulin rather than PRO195 polypeptide. Results are scored as positive in the assay if the uptake is greater than 1.5 times (stimulatory) or less than 0.5 time (inhibitory) the uptake of the insulin control. As PRO195 resulted in less than 0.5 the uptake of the insulin control, PRO195 tested positive as an inhibitor of glucose/FFA uptake in adipocyte cells.

The glucose/FFA uptake assay as described in Example 117 of the instant application was also well known in the art at the time of the effective filing date of the instant application. Similar assays were commonly used to identify potential anti-diabetic agents and study the regulatory mechanisms of important molecules involved in fat cell metabolism.

For example, at the time of the effective filing date of the instant application, it was well known in the art that increasing glucose uptake by adipocyte cells is a hallmark of a number of therapeutically effective agents, such as troglitazone and pioglitazone. (Tafuri, *Endocrinology*, 137(11): 4706-4712 (1996); Sandouk, *et al., Endocrinology*, 133(1):352-359 (1993); submitted with Appellants' Response filed December 30, 2004). Both troglitazone and pioglitazone are members of the thiazolidinedione class of compounds and have been used to effectively treat noninusulin-dependent diabetes mellitus (NIDDM), the most common form of diabetes. Both compounds function, at least in part, by increasing the number of cellular glucose transporters in order to facilitate increased glucose uptake.

Further, at the time of the effective filing date of the instant application, vanadium salts were considered as a possible treatment for diabetes, and several clinical trials had already been performed. (page 26617, right column, Goldwaser *et al.*, *J. Biol Chem.*, 274(37):26617-26624 (1999); submitted with Appellants' Response filed December 30, 2004). Using the rat adipocyte culture system similar to the system disclosed in the instant application, Goldwaser *et al.*, showed that vanadium ligand l-Glu (γ)HXM potentiates the capacity of free vanadium ions to activate glucose uptake and glucose metabolism in rat adipocytes *in vitro* by 4-5 folds and to lower blood glucose levels in hyperglycemic rats *in vivo* by 5-7 folds. This is further evidence that at the effective filing date of the present application one skilled in the art would have reasonably expected that molecules activating glucose uptake would find utility in the treatment of diabetes and related diseases.

In addition, the investigators in Mueller *et al.*, who were interested in determining the influence of glucose uptake on leptin secretion, employed essentially the same assay to measure changes in glucose uptake after insulin exposure. (Mueller *et al., Endocrinology*, 139(2): 551-558 (1998); submitted with Appellants' Response filed December 30, 2004). Figure 1A shows the glucose concentrations in medium from 0-96 hours from isolated rat adipocytes in primary culture with various insulin concentrations. As indicated by the decrease in glucose in the medium in Figure 1A, Mueller *et al.* suggest that insulin produced a concentration-dependent increase in glucose uptake by the cultured adipocytes. Based on these experimental results, the authors stated that insulin increased leptin secretion over 96 hours, and the increase in leptin was more closely related to the amount of glucose taken up by the adipocytes than to the insulin

concentration, suggesting a role for glucose transport and/or metabolism in regulating leptin secretion. (See Abstract).

Using the same assay system, Mueller *et al.* further studied the effect on leptin secretion of two well-known anti-diabetic agents, metformin and vanadium, which were known to enhance glucose uptake. (Muller *et al.*, *Obesity Research,* 8(7): 530-539 (2000); submitted with Appellants' Response filed December 30, 2004). The experimental data indicated that both metformin and vanadium increased glucose uptake and inhibited leptin secretion from cultured adipocytes.

Accordingly, Appellants respectfully submit that at the effective filing date of the instant application, one of skill in the art would have reasonably accepted that various compounds, such as PRO195, that are capable of modulating glucose uptake have a substantial, practical, real life utility. The above-mentioned studies have clearly established that the glucose/FFA uptake assay as described in the instant application is a reliable assay system to identify therapeutic agents for treating diseases and conditions such as obesity, diabetes, hyper- or hypo-insulinemia. Therefore, Appellants respectfully submit that a variety of real-life utilities, such as treatments for glucose uptake related diseases, including obesity and diabetes, are envisioned for PRO195 based on the glucose/FFA uptake assay results disclosed herein.

C. A *prima facie* case of lack of utility has not been established

The Examiner has asserted that "PRO195 was found to inhibit glucose/free fatty acid uptake. No evidence is offered as to how an inhibitor in such an assay should be used." The Examiner further asserted that "it would be contrary that such inhibitory compounds would be therapeutically beneficial in treating any specific disease. Notably, inhibition of glucose/free fatty acid uptake is contrary to and works in the opposite direction to therapeutics related to insulin resistance and/or diabetes." The Examiner concluded that "the references and evidence via the cited references fails to note specific and substantial utility that is either asserted or well established and recognized in the art based upon uptake .5 that of insulin" (Page 6 of the Office Action mailed April 12, 2005).

Appellants respectfully point out that the fact that PRO195 inhibits glucose uptake does not make it useless in such treatment. One of skill in the art would readily understand that a protein which inhibits glucose uptake into adipocytes is a useful therapeutic target, since

blocking the function of this protein would decrease the inhibition, and thus increase glucose uptake into adipocytes. Accordingly, the claimed PRO195 polypeptides are useful in the therapeutic treatment of disorders wherein stimulation of glucose uptake by adipocytes is expected to be therapeutically effective, such as obesity, diabetes, and hyper- or hypo-insulinemia.

Appellants also point out that Mueller *et al.* (1998) disclose that inhibitors of adipocyte glucose uptake, including 2-DG, phloretin, and cytocholasin B, inhibit leptin gene expression and leptin secretion from adipocytes. It was known in the art at the time of filing that leptin is involved in the regulation of food intake, energy expenditure, and body fat stores, and that leptin decreases after fasting or caloric restriction and increases a number of hours after refeeding. (Mueller *et al.* (1998), p. 551, col. 1). One of skill in the art would therefore have understood that agents capable of modulating leptin regulation would be useful in investigations regarding the treatment of obesity. Similarly, PRO195, as an inhibitor of adipocyte glucose uptake, would be useful as a pharmacological tool for investigation of leptin regulation and obesity, in the same way as agents already known and used in the art such as 2-DG, phloretin, and cytocholasin B.

1 The utility for PRO195 is based upon a specific, demonstrated activity and requires no further research or investigation

One of skill in the art would understand, as the Examiner acknowledges, that increasing glucose transport would be beneficial to the treatment of diabetes. PRO195 was demonstrated to be an inhibitor of glucose uptake, as shown in Example 117. Inhibiting the function of PRO195 would therefore increase glucose uptake. The specification has also described antagonists of PRO195 function, for example, antibodies specific for PRO195. See the specification at, for example, page 198, lines 3-6. Methods of making antibodies to PRO195 are disclosed at, for example, Example 104 and pages 217-219 of the specification, and methods of testing such antibodies for antagonist activity are disclosed at, for example, pages 196-199 of the specification. No further research or investigation is required to show that PRO195 is an inhibitor of glucose uptake, and that its function can be inhibited by molecules such as inhibitory antibodies. Accordingly, one of ordinary skill in the art would understand that inhibitors of PRO195, such as the disclosed antibodies, could be used in the treatment of disorders for which increased glucose uptake by adipocytes would be beneficial, such as diabetes, obesity, and hyper-

and hypo-insulinemia, and would understand exactly how to make and use these inhibitors, without any undue experimentation.

Appellants note that while an asserted utility must be described with specificity, the patent applicant need not demonstrate utility to a certainty. In *Stiftung v. Renishaw PLC,*[18] the United States Court of Appeals for the Federal Circuit explained, "An invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications." The Court has further explained that "the fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility."[19]

The specificity requirement is not, therefore, an onerous one. The specificity requirement is met unless the asserted utility amounts to a "nebulous expression" such as "biological activity" or "biological properties" that does not convey meaningful information about the utility of what is being claimed.[20] Such is clearly not the case here. The asserted utility for the claimed polypeptides is not based upon vague "biological properties," but a specific activity, inhibition of glucose uptake by adipocytes. This activity has already been demonstrated, as shown in Example 149 of the specification. Accordingly, PRO195, or antibodies thereto, have a specific utility in the treatment of disorders for which increased glucose uptake by adipocytes would be beneficial, such as diabetes, obesity, and hyper- and hypo-insulinemia.

2. The uses of PRO195 in the study of diabetes, obesity, and hyper- and hypo-insulinemia are practical uses beyond mere study of the invention itself

Appellants respectfully submit that the asserted uses for PRO195 in the investigation of leptin regulation is for the purpose of further characterizing not PRO195 itself, but the processes and diseases associated with glucose transport by adipocytes. PRO195, as an inhibitor of adipocyte glucose uptake, is useful as a pharmacological tool for investigation of leptin regulation and obesity, in the same way as agents already known and used in the art such as 2-DG, phloretin, and cytocholasin B. The rejection of the claims at issue is tantamount to an

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[18] *Stiftung v. Renishaw PLC,* 945 F.2d 1173, 1180, 20 U.S.P.Q.2d 1094 (Fed. Cir. 1991).

[19] *Envirotech Corp. v. Al George, Inc.,* 730 F.2d 753, 762, 221 U.S.P.Q. 473, 480 (Fed. Cir. 1984).

[20] *Cross* v. *Iizuka,* 753 F.2d 1040, 1048 (Fed. Cir. 1985).

assertion that the use of an invention as tool for research is not a "substantial" use. Because the rejection assumes a substantial overstatement of the law, and is incorrect in fact, it must be withdrawn.

There is no authority for the proposition that use as a tool for research is not a substantial utility. Indeed, the Patent Office itself has recognized that just because an invention is used in a research setting does not mean that it lacks utility:

> Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the specific invention is in fact "useful" in a patent sense. Instead, Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm.[21]

The PTO's actual practice has been consistent with that approach. It has routinely issued patents for inventions whose only use is to facilitate research, such as DNA ligases, acknowledged by the PTO's Training Materials to be useful.

The subset of research uses that are not "substantial" utilities is limited. It consists only of those uses in which the claimed invention is to be an **object** of further study, thus merely inviting further research on the invention itself. This follows from *Brenner*, in which the U.S. Supreme Court held that a process for making a compound does not confer a substantial benefit where the only known use of the compound was to be the object of further research to determine its use.[22]

Beneficial uses beyond studying the claimed invention itself have been demonstrated for PRO195, in particular, the study of disorders associated with altered glucose uptake by adipocytes, such as diabetes, obesity, and hyper- and hypo-insulinemia. Accordingly, the claimed PRO195 polypeptides have a specific and substantial utility.

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[21] M.P.E.P §2107.01 I(C)

[22] *Brenner v. Manson,* 383 U.S. 519, 148 U.S.P.Q. (BNA) 689 (1966).

Appellants reiterate that, as discussed above, the Examiner must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of the statement of utility. Only after the Examiner made a proper *prima facie* showing of lack of utility, does the burden of rebuttal shift to the applicant. The issue will then be decided on the totality of evidence. Therefore, the legal standard for patentable utility is not absolute certainty. Appellants submit that clear evidence supports the glucose uptake inhibition activity of PRO195. No evidence has been presented to make it more likely than not that one of skill in the art would doubt the truth of this asserted utility of PRO195 as an inhibitor of glucose uptake.

Accordingly, Appellants respectfully submit that at the effective filing date of the instant application, one of skill in the art would have reasonably accepted that various compounds, such as PRO195, that are capable of modulating glucose uptake have a substantial, practical, real life utility. The above-mentioned studies have clearly established that the glucose/FFA uptake assay as described in the instant application is a reliable assay system to identify therapeutic agents for treating diseases and conditions such as obesity, diabetes, hyper- or hypo-insulinemia, or pharmacological tools for the study of these diseases and conditions. Therefore, Appellants respectfully submit that a variety of real-life utilities, such as study and treatment of glucose uptake related diseases, including obesity and diabetes, are envisioned for the claimed PRO195 polypeptide based on the glucose/FFA uptake assay results disclosed herein.

For the reasons given above, Appellants respectfully submit that the present specification clearly describes, details and provides a patentable utility for the claimed invention. Accordingly, Appellants respectfully request reconsideration and reversal of the rejection of Claims 58-65 and 68-70 under 35 U.S.C. §101.

ISSUE II: Claims 58-65 and 68-70 satisfy the enablement requirement of 35 USC §112, first paragraph.

Claims 58-65 and 68-70 stand rejected under 35 U.S.C. §112, first paragraph, allegedly since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art would not know how to use the claimed invention.

In this regard, Appellants refer to the arguments and information presented above in response to the outstanding rejection under 35 U.S.C. § 101, wherein those arguments are

incorporated by reference herein. Appellants respectfully submit that as described above, the PRO195 polypeptides have utility in the treatment of disorders for which modulation of glucose uptake by adipocytes would be beneficial, such as obesity, diabetes, and hyper- or hypo-insulinemia, or as pharmacological tools for the study of these diseases and conditions, and based on such a utility, one of skill in the art would know exactly how to use the claimed polypeptides without undue experimentation.

Claims 58-65 and 68-70 further stand rejected under 35 USC § 112, first paragraph, as failing to enable a person of skill in the art to make and use the invention commensurate in scope with the claims. In particular, the Examiner asserts that "the specification does not reasonably provide enablement for the variable peptide sequences and for such generic sequences where no requisite functional activity is provided as claimed." (See page 10 of the Office Action mailed April 12, 2005).

A. The Legal Test for Enablement

The test of enablement is whether one skilled in the art could make and use the claimed invention from the disclosure provided by Appellants coupled with information known in the art at the time the invention was made, without undue experimentation.[23,24] Accordingly, the test for enablement is not whether any experimentation is necessary, but whether, if experimentation is required, it is undue.[25] The mere fact that an extended period of experimentation is necessary does not make such experimentation undue.[26,27]

A finding of lack of enablement and a determination that undue experimentation is necessary requires an analysis of a variety of factors *(i.e.,* the *In re* Wands factors). The most important factors that must be considered in this case include: 1) the nature of the invention; 2) the level of one of ordinary skill in the art; 3) guidance provided in the specification, 4) the state of the prior art, and 8) the breadth of the claims.

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[23] M.P.E.P. §2164.01.

[24] *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 U.S.P.Q.2d 1217, 1223 (Fed. Cir. 1998)).

[25] *In re Angstadt*, 537 F.2d 498, 504, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976).

[26] *In re Colianni*, 561 F.2d 220, 224, 195 U.S.P.Q. 150, 153 (C.C.P.A. 1977).

[27] M.P.E.P. §2164.06.

"How a teaching is set forth, by specific example or broad terminology, is not important."[28,29] Limitations and examples in the specification do not generally limit what is covered by the claims" M.P.E.P. § 2164.08. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. It is well settled that patent Appellants are not required to disclose every species encompassed by their claims, even in an unpredictable art. The legal standard merely requires that there must be sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and use the invention as broadly as it is claimed.[30]

B. The specification provides sufficient information to enable the claimed invention

Appellants respectfully submit that Claim 63 claims the full-length polypeptide of SEQ ID NO:330, with or without its signal peptide sequence. As disclosed, for example, in Figure 132, the signal peptide sequence of SEQ ID NO:330 comprises amino acid residues 1-31. Neither Claim 63 nor any other claim recites the extracellular domain of the polypeptide. Appellants have clearly provided the full-length sequence of SEQ ID NO:330 for the PRO195 polypeptide, thus one skilled in the art would easily know how to make the polypeptide, with or without the identified signal peptide sequence. In addition, as mentioned above, PRO195 was demonstrated to inhibit the uptake of glucose or FFA by adipocyte cells. Therefore, based on this information one skilled in the art would have known at the time of filing how to use the full-length PRO195 polypeptide (SEQ ID NO:330), with or without its signal peptide sequence, in the therapeutic treatment of disorders wherein stimulation of glucose uptake by adipocytes is expected to be therapeutically effective, such as obesity, diabetes, and hyper- or hypo-insulinemia, or as a pharmacological tool for the study of these diseases and conditions..

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[28] M.P.E..P. §2164.08.

[29] *In re Marzocchi*, 439 F.2d 220, 223-4, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971).

[30] *Enzo Biochem., Inc. v. Calgene, Inc.*, 188 F.3d 1362, 1372 (Fed. Cir. 1999) (quoting *In re Vaeck*, 947 F.2d 488, 496 (Fed. Cir. 1991)).

Accordingly, Claim 63 (and, as a consequence, those claims dependent from the same) meets the enablement requirement of 35 U.S.C. §112, first paragraph.

Appellants have provided native PRO195 sequence SEQ ID NO:330. The present application also describes methods for identifying polypeptides which inhibit the uptake of glucose or FFA by adipocyte cells. Example 117 of the present application provides a detailed protocol for the adipocyte glucose/FFA uptake assay. By following the disclosure in the specification, one skilled in the art can easily test whether a variant PRO195 protein is an inhibitor of glucose uptake. The specification further describes methods for the determination of percent identity between two amino acid sequences. (See page 123, line 24 to page 125, line 14). In fact, the specification teaches specific parameters to be associated with the term "percent identity" as applied to the present invention. Accordingly, one of skill in the art could identify whether the variant PRO195 native sequence falls within the parameters of the claimed invention. Once such an amino acid sequence was identified, the specification sets forth methods for making the amino acid sequences (see page 180, line 9 to page 184, line 35) and methods of preparing the PRO polypeptides (see page 184, line 37 and onward).

Therefore, Appellants respectfully submit that one of skill in the art could readily test a variant polypeptide to determine whether it inhibits the uptake of glucose or FFA by adipocyte cells by the methods set forth in Example 117. Furthermore, one of ordinary skill in the art has a sufficiently high level of technical competence to identify sequences with at least 80% identity to SEQ ID NO:330. Accordingly, one of ordinary skill could practice the claimed invention without undue experimentation.

The Examiner has asserted that the specification "does not enable this broad scope of the claims that encompasses a multitude of analogs or equivalents because the specification does not teach which residues can or should be modified such that the polypeptides retain sufficient structural similarity to evoke activity." (Page 11 of the Office Action mailed April 12, 2005). Appellants respectfully disagree. Appellants respectfully point out that the specification provides detailed guidance as to changes that may be made to a PRO polypeptide without adversely affecting its activity (page 180, line 9 to page 183, line 8). This guidance includes a listing of exemplary and preferred substitutions for each of the twenty naturally occurring amino acids (Table 6, page 182). Furthermore, the claims require that the polypeptide variants retain the

functional activity of PRO195, and the specification provides an assay for this activity. Thus the specification has provided all the guidance needed to permit one of skill in the art to make and use the claimed variant native sequence polypeptides.

The Examiner has asserted that "protein chemistry is an unpredictable art of biotechnology," because "replacement of single amino acid residues may lead to both structural and functional changes in biological activity and immunological recognition." (Page 10 of the Office Action mailed April 12, 2005). In support of this assertion, the Examiner cited articles by Skolnick *et al.* and Jobling *et al.*

Skolnick *et al.* is a review article computational predictions of protein structure and function from sequence data. The authors are concerned with structural and functional predictions for unknown proteins, and say nothing about the effects of single amino acid substitutions on the function of known proteins.

Jobling *et al.* describe site directed mutagenesis of the B subunit of cholera toxin. The authors made mutations at sites already known to be important for protein structure or function, including known binding site residues, and two cysteine residues involved in a structurally important disulfide bond (pages 1755-1756). Given that the sites of mutagenesis were specifically selected as being important for protein structure or function, it is noteworthy that in most cases many substitutions could be made that had no effect on protein structure or function. Those substitutions that did alter the protein's activity were highly non-conservative substitutions, such as the replacement of glycine with charged or large hydrophobic amino acids, or the substitution of arginine with negatively charged amino acids. Thus Jobling *et al.* confirm that most single amino acid changes, particularly conservative changes, do not affect protein structure or function.

Accordingly, one of ordinary skill in the art would be able to use the guidance provided in the specification, including the listing of conservative amino acid substitutions provided in Table 6, to make nucleic acids encoding variants of SEQ ID NO:330 that would be expected to retain the activity of SEQ ID NO:330 in inhibiting glucose/FFA uptake by adipocytes. In addition to this guidance, the specification also provides an adipocyte glucose/FFA uptake assay, as disclosed in Example 117. It would be a simple matter for one skilled in the art to test the polypeptides to see if they inhibit the uptake of glucose or FFA by adipocyte cells using the methods of Example 117. This would not require undue experimentation.

The claims currently recite peptide sequences associated with a biological activity. This biological activity together with the well defined relatively high degree of sequence identity and general knowledge in the art at the time the invention was made, sufficiently defines the claimed genus such that, one skilled in the art, at the effective date of the present application, would have known how to make and use the claimed polypeptide sequences without undue experimentation. As the M.P.E.P. states, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation."[31]

As discussed above, a considerable amount of experimentation is permissible, if it is merely routine. Appellants submit that the identification of variant PRO195 polypeptides having at least 80% identity to SEQ ID NO:330 wherein the polypeptide inhibits the uptake of glucose or FFA by adipocyte cells can be performed by techniques that were well known in the art at the priority date of this application, and that the performance of such work does not require undue experimentation.

Accordingly, Appellants respectfully request reconsideration and reversal of the enablement rejection of Claims 58-65 and 68-70 under 35 U.S.C. §112, first paragraph.

ISSUE III: Claims 58-65 and 68-70 satisfy the written description requirement of 35 U.S.C. §112, First Paragraph

Claims 58-65 and 68-70 stand rejected under 35 U.S.C. §112, first paragraph as allegedly lacking adequate written description. In particular, the Examiner has asserted that "the claims as written include polypeptides having at least 80-99% sequence identity with SEQ ID NO:330 and polypeptides including or lacking various regions including, lacking its signal peptide, the extracellular domain, the extracellular domain but lacking its signal peptide, but for which no particular biological activity or function is recited." Therefore the Examiner asserted that "the instant disclosure of a single polypeptide, that of SEQ ID NO:330 with the instantly disclosed specific activities, does not adequately support the scope of the claimed genus, which encompasses a substantial variety of subgenera." (Page 7 of the Office Action mailed April 12, 2005).

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[31] M.P.E.P. §2164.01 citing *In re Certain Limited-charge Cell Culture Microcarriers*, 221 U.S.P.Q. 1165, 1174 (Int'l Trade Comm'n 1983), *aff' sub nom. Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 U.S.P.Q. 428 (Fed. Cir. 1985).

A. The Legal Test for Written Description

The well-established test for sufficiency of support under the written description requirement of 35 U.S.C. §112, first paragraph, is "whether the disclosure of the application as originally filed reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter, rather than the presence or absence of literal support in the specification for the claim language."[32,33] The adequacy of written description support is a factual issue and is to be determined on a case-by-case basis.[34] The factual determination in a written description analysis depends on the nature of the invention and the amount of knowledge imparted to those skilled in the art by the disclosure.[35,36]

In *Environmental Designs, Ltd. v. Union Oil Co.*,[37] the Federal Circuit held, "Factors that may be considered in determining level of ordinary skill in the art include: (1) the educational level of the inventor; (2) type of problems encountered in the art; (3) prior art solutions to those problems; (4) rapidity with which innovations are made; (5) sophistication of the technology; and (6) educational level of active workers in the field." (Emphasis added).[38] Further, The "hypothetical 'person having ordinary skill in the art' to which the claimed subject matter pertains would, of necessity have the capability of understanding the scientific and engineering principles applicable to the pertinent art."[39,40].

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[32] *In re Kaslow*, 707 F.2d 1366, 1374, 212 U.S.P.Q. 1089, 1096 (Fed. Cir. 1983).

[33] *See also Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 U.S.P.Q.2d at 1116 (Fed. Cir. 1991).

[34] *See e.g., Vas-Cath*, 935 F.2d at 1563; 19 U.S.P.Q.2d at 1116.

[35] *Union Oil v. Atlantic Richfield Co.*, 208 F.2d 989, 996 (Fed. Cir. 2000).

[36] *See also* M.P.E..P. §2163 II(A).

[37] 713 F.2d 693, 696, 218 U.S.P.Q. 865, 868 (Fed. Cir. 1983), *cert. denied*, 464 U.S. 1043 (1984).

[38] *See also* M.P.E.P. §2141.03.

[39] *Ex parte Hiyamizu*, 10 U.S.P.Q.2d 1393, 1394 (Bd. Pat. App. & Inter. 1988) (emphasis added).

[40] See *also* M.P.E.P. §2141.03.

B. The specification provides sufficient written description for the claimed invention

Appellants respectfully submit that Claim 63 (and, as a consequence, those claims dependent from the same), directed to the polypeptide of SEQ ID NO:330, with or without its signal peptide sequence, meets the written description requirement under 35 U.S.C. §112, first paragraph. The Examiner has acknowledged that Appellants have described the polypeptide sequence of SEQ ID NO:330. (Page 7 of the Office Action mailed April 12, 2005). As disclosed, for example, in Figure 132, the signal peptide sequence of SEQ ID NO:330 comprises amino acid residues 1-31. Neither Claim 63 nor any other claim recites the extracellular domain of the polypeptide. Accordingly, the specification has clearly described the polypeptide of SEQ ID NO:330, with or without its signal peptide, and thus the subject matter of Claims 63-65 and 68 meets the written description requirement under 35 U.S.C. §112, first paragraph.

Appellants further submit that the instant specification evidences the actual reduction to practice of a full-length PRO195 polypeptide of SEQ ID NO:330, with or without its signal peptide sequence. As stated above, the Examiner has acknowledged that a polypeptide comprising the sequence set forth in SEQ ID NO:330 meets the written description provision of 35 U.S.C. §112, first paragraph. Thus, the genus of polypeptides with at least 80% sequence identity to SEQ ID NO:330, which possess the functional property of inhibiting the uptake of glucose or FFA by adipocyte cells would meet the requirement of 35 U.S.C. §112, first paragraph, as providing adequate written description.

The specification describes methods for the determination of percent identity between two amino acid sequences. (See page 123, line 24 to page 125, line 14). In fact, the specification teaches specific parameters to be associated with the term "percent identity" as applied to the present invention. The specification further provides detailed guidance as to changes that may be made to a PRO polypeptide without adversely affecting its activity (page 180, line 9 to page 183, line 8). This guidance includes a listing of exemplary and preferred substitutions for each of the twenty naturally occurring amino acids (Table 6, page 182). The specification describes methods for one of ordinary skill in the art to identify polypeptides having at least 80% identity to SEQ ID NO:330 wherein the polypeptide inhibits the uptake of glucose or FFA by adipocyte cells. Specifically, Example 117 sets forth an assay for determining whether a polypeptide having at

least 80% identity to PRO195 inhibits the uptake of glucose or FFA by adipocyte cells. Thus one of ordinary skill in the art would have understood at the time of filing what was encompassed by the claims.

The Examiner has asserted that "the specification provides only a single sequence which provides for such inhibition and fails to describe what other sequences inhibit," and that "there is no description or guidance as to how similar the other related sequence need to be such that the variability in structure correlates to inhibition or some other known and useful function." (Page 9 of the Office Action mailed April 12, 2005). As discussed above, the specification provides detailed guidance as to changes that may be made to a PRO polypeptide without adversely affecting its activity, including a listing of exemplary and preferred substitutions for each of the twenty naturally occurring amino acids (Table 6, page 182). Furthermore, the claims require that the polypeptide variants retain the functional activity of PRO195, and the specification provides an assay for this activity. Thus the specification has provided all the guidance needed to permit one of skill in the art to understand what is encompassed by the claimed genus of polypeptide variants, and that Appellants were in possession of the claimed genus of polypeptide variants at the time of filing.

The Examiner has asserted that "the instant disclosure of a single polypeptide, that of SEQ ID NO:330 with the instantly disclosed activities, does not adequately support the scope of the claimed genus." (Page 7 of the Office Action mailed April 12, 2005). In support of this assertion, the Examiner cited cases including *Regents of the University of California v. Eli Lilly & Co.* and *Fiers v. Revel* and *Amgen v. Chugai*. (Pages 7-8 of the Office Action mailed April 12, 2005).

Appellants submit that *Fiers v. Revel* and *Amgen v. Chugai* addressed conception and the written description requirement in the context of DNA-related inventions. The *Amgen* court held that conception of a DNA invention "has not been achieved until reduction to practice has occurred, *i.e.*, until after the gene has been isolated." 927 F.2d 1200 (Fed. Cir.), *cert. denied*, 502 U.S. 856 (1991), at 1206. The *Fiers* court extended this decision into the written description arena, holding that "[i]f a conception of a DNA requires a precise definition, such as by structure, formula, chemical name, or physical properties, as we have held, then a description also requires

that degree of specificity." *Fiers*, 984 F.2d at 1171. Since the instant claims are directed to polypeptides, *Fiers* and *Amgen* are distinguished on the facts and do not apply.

More recently, in *Enzo Biochem., Inc. v. Genprobe, Inc.* 296 F.3d 1316 (Fed. Cir. 2002), the court adopted the standard that "the written description requirement can be met by 'showing that the invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics, . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." *Id.* at 1324. While the invention in *Enzo* was still a DNA, the holding has been treated as being applicable to proteins as well. Indeed, the court adopted the standard from the USPTO's Written Description Examination Guidelines, which apply to both proteins and nucleic acids.

Accordingly, current applicable case law holds that biological sequences are not adequately described **solely** by a description of their desired functional activities. The instant claims meet the standard set by the *Enzo* court in that the claimed sequences are defined not only by functional properties, but also by structural limitations. It is well established that a **combination** of functional and structural features may suffice to describe a claimed genus. "An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics."[41] As discussed above, Appellants have recited structural features, namely, 80% sequence identity to SEQ ID NO:330, which are common to the genus. The genus of claimed polypeptides is further defined by having a specific activity, inhibition of the glucose and/or FFA uptake by adipocyte cells. Accordingly, a description of the claimed genus has been achieved.

This particular combination of functional activity and structural homology, as disclosed in the specification, has been recognized by the USPTO as sufficient to describe a claimed genus of polypeptides. The Board's attention is respectfully directed to Example 14 of the Synopsis of Application of Written Description Guidelines issued by the U.S. Patent Office, which clearly

[41] M.P.E.P. §2163 II(A)(3)(a)

states that protein variants meet the requirements of 35 U.S.C. §112, first paragraph, as providing adequate written description for the claimed invention even if the specification contemplates but does not exemplify variants of the protein if (1) the procedures for making such variant proteins are routine in the art, (2) the specification provides an assay for detecting the functional activity of the protein and (3) the variant proteins possess the specified functional activity and at least 95% sequence identity to the reference sequence.

As discussed above, the procedures for making the claimed variant polypeptides are well known in the art and described in the specification. The specification also provides an assay, shown in Example 117, for detecting the recited functional activity of the variant polypeptides. Finally, the claimed variant polypeptides possess both the specified functional activity and a defined degree of sequence identity to the reference sequence, SEQ ID NO:330. Accordingly, the claimed polypeptide variants meet the standards set forth in the Written Description Guidelines.

Thus the specification provides adequate written description for polypeptides having at least 80% identity to SEQ ID NO:330 wherein the polypeptide inhibits the uptake of glucose or FFA by adipocyte cells. Appellants therefore respectfully request reconsideration and reversal of the written description rejection of Claims 58-65 and 68-70 under 35 U.S.C. §112, first paragraph.

CONCLUSION

For the reasons given above, Appellants submit that the adipocyte glucose/FFA uptake assay disclosed in Example 117 of the specification provides at least one asserted specific and substantial patentable utility for the claimed PRO195 polypeptides, and that one of ordinary skill in the art would accept this asserted utility as credible and would understand how to make and use the claimed polypeptides. Therefore, claims 58-65 and 68-70 meet the requirements of 35 U.S.C. §101 and 35 U.S.C. §112, first paragraph.

Appellants further submit that Claims 58-65 and 68-70 meet the written description requirement of 35 U.S.C. §112, first paragraph.

Accordingly, reversal of all the rejections of Claims 58-65 and 68-70 is respectfully requested.

Please charge any additional fees, including fees for additional extension of time, or credit overpayment to Deposit Account No. **08-1641** (referencing Attorney's Docket No. **39780-2630 P1C3**).

Respectfully submitted,

Date: November 10, 2005

By: _____
Barrie D. Greene (Reg. No. 46,740)

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8. CLAIMS APPENDIX

Claims on Appeal

58. An isolated polypeptide having at least 80% amino acid sequence identity to:

(a) the amino acid sequence of the polypeptide of SEQ ID NO:330;

(b) the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or

(c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772,

wherein the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells.

59. The isolated polypeptide of Claim 58 having at least 85% amino acid sequence identity to:

(a) the amino acid sequence of the polypeptide of SEQ ID NO:330;

(b) the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or

(c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772,

wherein the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells.

60. The isolated polypeptide of Claim 58 having at least 90% amino acid sequence identity to:

(a) the amino acid sequence of the polypeptide of SEQ ID NO:330;

(b) the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or

(c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772,

wherein the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells.

61. The isolated polypeptide of Claim 58 having at least 95% amino acid sequence identity to:

(a) the amino acid sequence of the polypeptide of SEQ ID NO:330;

(b) the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or

(c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772,

wherein the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells.

62. The isolated polypeptide of Claim 58 having at least 99% amino acid sequence identity to:

(a) the amino acid sequence of the polypeptide of SEQ ID NO:330;

(b) the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or

(c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772,

wherein the polypeptide inhibits the uptake of glucose or FFA (free fatty acid) by adipocyte cells.

63. An isolated polypeptide comprising:

(a) the amino acid sequence of the polypeptide of SEQ ID NO:330;

(b) the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide; or

(c) the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772.

64. The isolated polypeptide of Claim 63 comprising the amino acid sequence of the polypeptide of SEQ ID NO:330.

65. The isolated polypeptide of Claim 63 comprising the amino acid sequence of the polypeptide of SEQ ID NO:330, lacking its associated signal peptide.

68. The isolated polypeptide of Claim 63 comprising the amino acid sequence of the polypeptide encoded by the full-length coding sequence of the cDNA deposited under ATCC accession number 209772.

69. A chimeric polypeptide comprising a polypeptide according to Claim 58 fused to a heterologous polypeptide.

70. The chimeric polypeptide of Claim 69, wherein said heterologous polypeptide is an epitope tag or an Fc region of an immunoglobulin.

9. EVIDENCE APPENDIX

1. Tafuri, S.R., "Troglitazone Enhances Differentiation, Basal Glucose Uptake, and Glut 1 Protein Levels in 3T3-L1 Adipocytes," Endocrinology, 137:4706-4712 (1996).

2. Sandouk, T., et al., "The Antidiabetic Agent Pioglitazone Increases Expression of Glucose Transporters in 3T3-F442A Cells by Increasing Messenger Ribonucleic Acid Transcript Stability", Endocrinology 133:352-359 (1993).

3. Goldwaser, I., et al., "L-Glutamic Acid γ-Monohydroxamate: A potentiator of vanadium-evoked glucose metabolism *in vitro* and *in vivo*", J. Biol. Chem. 274:26617-26624 (1999).

4. Mueller, W. M., et al., "Evidence That Glucose Metabolism Regulates Leptin Secretion from Cultured Rat Adipocytes", Endocrinology 139:551-558 (1998).

5. Mueller, W. M., et al., "Effects of Metformin and Vanadium on Leptin Secretion from Cultured Rat Adipocytes", Obesity Research 8:530-539 (2000).

6. Skolnick, J. et al., "From genes to protein structure and function: novel applications of computational approaches in the genomic era," *Trends Biotechnol.* **18**:34-39 (2000).

7. Jobling, M. G. et al, "Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis," *Mol Microbiol.* **5**:1755-1767 (1991).

Items 1-5 were made of record by Appellants in their IDS filed December 30, 2004, and were initialed as considered by the Examiner on March 30, 2005.

Items 6-7 were made of record by the Examiner in the Office Action mailed June 30, 2004.

10. RELATED PROCEEDINGS APPENDIX

None.

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Troglitazone Enhances Differentiation, Basal Glucose Uptake, and Glut1 Protein Levels in 3T3-L1 Adipocytes

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ABSTRACT

Troglitazone is a member of the thiazolidinedione class of compounds, which act as insulin-sensitizing agents when administered to human patients and animal models displaying noninsulin-dependent diabetes mellitus. In Zucker rats, the antidiabetic activity is associated with increased glucose uptake in adipose tissue. To understand the direct effects troglitazone has on adipocyte metabolism, 3T3-L1 preadipocytes and adipocytes were treated with the compound. The addition of troglitazone enhanced the rate and percent differentiation of fibroblasts to adipocytes. Northern analysis indicated that during differentiation, expression of the adipocyte-specific transcription factor, CCAAT enhancer binding protein-α, increased more rapidly in troglitazone-treated cells, but did not change in fully differentiated adipocytes. To assess the metabolic consequences of troglitazone treatment, both basal and insulin-stimulated glucose uptake were monitored in treated cells. Troglitazone treatment increased basal glucose transport 1.5- to 2.0-fold, whereas insulin-stimulated uptake was unaffected. Enhanced basal transport was caused by an increased synthesis of both Glut1 glucose transporter messenger RNA and protein. These results suggest the possibility that *in vivo*, the troglitazone-dependent increase in glucose disposal may be attributable in part to modifications in the expression of Glut1 in insulin-responsive tissues. (*Endocrinology* **137:** 4706–4712, 1996)

NONINSULIN-DEPENDENT diabetes mellitus (NIDDM), the most common form of diabetes, is estimated to affect more than 4 million people in the United States (1). This disease commonly develops during middle age and is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance. At present, treatment consists of behavioral modifications in conjunction with the administration of insulin and oral hypoglycemic agents (sulfonylureas and biguanide compounds). However, these treatments often fail to ameliorate one of the main underlying causes of the disease, insulin resistance. The thiazolidinediones, a new class of compounds, differ markedly from other antidiabetic agents in that they decrease hyperglycemia and hyperinsulinemia by increasing insulin sensitivity in target tissues. These compounds increase peripheral glucose uptake while decreasing insulin secretion and gluconeogenesis in a wide variety of type II animal models (2–4). Troglitazone, a member of this compound class, improves glucose tolerance and insulin sensitivity in both diabetic (5, 6) and glucose-intolerant (7) patients. Little is known about the biochemical mechanism of action of these compounds.

Adipose tissue is highly responsive to insulin. Its primary role is to store energy when nutrients are plentiful and to release energy during fasting and starvation. Adipose tissue also plays a pivotal role in metabolic homeostasis. Adipose tissue is responsible for 50–70% of lactate production in the adult and consequently contributes to the regulation of glycogen synthesis and gluconeogenesis (8). Moreover, the recent discovery of the ob gene product indicates that adipose tissue secretes hormones capable of regulating feeding patterns, satiety, and adiposity (9). Because of the importance of adipose tissue in metabolic regulation and insulin resistance, it may play an important role in the mechanism of action of thiazolidinediones.

To understand if and how the thiazolidinediones affect adipose cell metabolism, a study was designed to determine how troglitazone affects glucose utilization in 3T3-L1 adipocytes. This system was chosen because it is easily manipulated and is not complicated by the problems associated with the metabolic feedback loops present *in vivo*. Additionally, previous experiments with pioglitazone, another member of the thiazolidinedione family, in 3T3-F442A adipose cells suggested that these compounds increase differentiation (10, 11) and glucose uptake (12) in adipocytes. In the studies discussed here, troglitazone increased differentiation in 3T3-L1 cells when administered at the initiation of the differentiation protocol. Furthermore, troglitazone enhanced glucose uptake in these cells by altering the total number of basal glucose transporters within the cell.

Materials and Methods

Materials

3T3-L1 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). High glucose DMEM culture medium and bovine serum were purchased from Life Technologies (Gaithersburg, MD). Insulin, dexamethasone, and isomethylbutylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). Troglitazone and pioglitazone were synthesized by Parke-Davis (Ann Arbor, MI) and Sankyo (Tokyo, Japan), respectively.

Cell culture

3T3-L1 fibroblasts were grown and passaged in DMEM containing 10% FBS. For adipocyte differentiation, 2 day postconfluent cells were placed in 10% FBS-DMEM, 1 μg/ml insulin, 0.25 μM dexamethasone,

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and 0.5 mM isomethylbutylxanthine. Forty-eight hours later, the medium was changed to 10% FBS-DMEM containing 1 μg/ml insulin, and after an additional 48 h, the medium was replaced with 10% FBS-DMEM. Thereafter, the medium was changed every 2 days. Troglitazone was dissolved in dimethylsulfoxide as a 1000-fold stock solution and administered at the initiation of differentiation and with every medium change unless otherwise indicated.

Glucose uptake

Basal glucose uptake was measured using a modified version of the hexose transport procedure described by de Herreros and Birnbaum (13). Briefly, cells were washed with PBS and placed in glucose-free DMEM containing 1% BSA for 30 min at 37 C. At this time, 1 μCi/ml [^{14}C]2-deoxyglucose and 1 mM glucose were added, and the cells were incubated for 30 min at 22 C. Subsequently, the cells were washed with PBS and 10 mM glucose at 4 C and lysed with 0.5 N NaOH. The lysates were neutralized with acetic acid and counted. To measure insulin-stimulated glucose uptake, cells were serum starved for 3 h before initiation of the assay. Stimulation with insulin (1×10^{-6} M) was initiated 15 min before the addition of labeled glucose.

RNA preparation, Northern blot analysis, and ribonuclease (RNase) protection assays

The guanidinium lysis method (Ultraspec RNA isolation system, Biotecx, Houston, TX) was used to prepare RNA samples. For Northern analysis, 15–30 μg RNA/sample were resolved on an 0.8–1% agarose formaldehyde gel and transferred to nitrocellulose. The blots were hybridized in 1 M NaCl, 1% SDS, and 10% dextran sulfate for 16–20 h at 42 C. Washes were conducted in 0.1 × SSC (standard saline citrate)-0.1% SDS at 50 C. The indicated complementary DNAs were labeled with [α-^{32}P]deoxy-CTP using the random prime method and used as probes. RNase protection assays were performed using the RNase A nuclease assay according to manufacturer's instructions (Ambion, Houston, TX). [α-^{32}P]UTP-labeled singled stranded RNA probes were generated using T3/T7 *in vitro* transcription procedures. All results were quantitated using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Western blot analysis

Cell lysates were prepared in HNTG buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and 1 mM EDTA). The Bio-Rad protein analysis system (Bio-Rad Laboratories, Richmond, CA) was used to determine protein content. Western blots were performed using standard protocols. Antibody interactions were detected using the chemiluminescent assay (ECL, Amersham, Arlington Heights, IL). The Glut1 antibody was produced using a glutathione *S*-transferase fusion protein encoding the last 39 amino acids of the Glut1 glucose transporter. The antibody was immunoaffinity purified and shown not to cross-react with purified Glut4 glucose transporter protein. The Glut4-specific antibody was kindly provided by Dr. Michael Mueckler.

Results

Troglitazone enhances adipocyte differentiation in 3T3-L1 cells

The differentiation of 3T3-L1 preadipocytes into adipocytes is a complex process that is affected by cell passage number and a variety of environmental conditions. Under ideal conditions, 95–100% of confluent preadipocytes can be converted into fat droplet-containing adipocytes in 5–7 days, with fat droplets first appearing on day 4. However, as the preadipocytes are passaged, the efficiency of differentiation diminishes in a clonal manner, such that a confluent plate will contain islands of adipocytes within undifferentiated fibroblasts. This loss of phenotype is also associated with a decrease in the rate of differentiation, causing the initial appearance of fat droplets to occur around day 5 or 6. Initial experiments were conducted using cells with an adipocyte conversion frequency of approximately 50%. To study the effects of troglitazone on differentiation, 0.5–5 μM compound was added with 167 nM insulin, 0.25 μM dexamethasone, and 0.5 mM isomethylbutylxanthine (hormone cocktail) at the initiation of differentiation and reapplied with each medium change. Eight-day troglitazone treatment without hormone cocktail showed minimal differentiation (Fig. 1, A and B). Less than 1% of the cells became adipocytes; however, the number of adipocytes in the drug-treated sample was greater than that in the untreated sample. In the presence of hormone cocktail, troglitazone significantly enhanced the percentage of adipocyte differentiation (Fig. 1, C and D). Nearly 100% of treated cells contained fat droplets compared to 50% of the untreated group. Additionally, the rate of differentiation was enhanced as fat droplets began to accumulate in the treated group 1 day before the control group.

Adipocyte differentiation has been shown to be dependent upon the activation of several transcription factors, which, in turn, initiate the expression of a repertoire of adipocyte genes. One such factor, CCAAT enhancer binding protein-α (C/EBPα), has been shown to be necessary and sufficient for adipocyte conversion in both preadipocyte and fibroblast cell lines (14–17). Moreover, mice lacking C/EBPα expression fail to accumulate both white and brown fat, suggesting that C/EBPα is required for terminal adipoctye differentiation (18). C/EBPα mRNA is not expressed in preadipocytes, is induced 2–3 days after the initiation of differentiation, and is maintained at a high level in the adipocyte (19). As troglitazone increases adipocyte differentiation, total RNA was isolated from differentiating cells treated with hormone cocktail containing 0 or 5 μM troglitazone and probed with labeled C/EBPα complementary DNA to determine whether troglitazone enhances C/EBPα expression. The control cells used in this experiment differentiated 95–100% without the

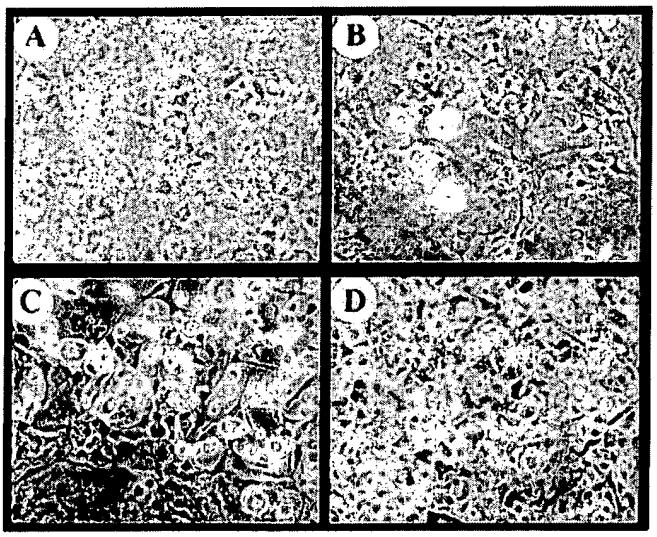

FIG. 1. Troglitazone enhances the differentiation of 3T3-L1 preadipocytes to adipocytes. Confluent preadipocytes were maintained in culture for 8 days without compound (A) or with 5 μM troglitazone (B), as described in *Materials and Methods*. Identical cells were treated with differentiation cocktail (167 nM insulin, 0.25 μM dexamethasone, and 0.5 mM isomethylbutylxanthine) with (D) or without (C) 5 μM troglitazone.

addition of drug; therefore, the ratio of adipocytes/preadipocytes in the drug-treated and untreated samples was identical. As shown in Fig. 2, troglitazone increased the rate of C/EBPα mRNA accumulation during differentiation, but did not affect the levels of C/EBPα message after differentiation was complete. At 48 h, treated cells contained 2.5 times the level of C/EBPα mRNA found in control cells. Conversely, the levels on day 6 were nearly identical in both samples. Multiple experiments demonstrated that the onset of C/EBPα expression varied with the passage number of the cells; however, in all cases troglitazone enhanced the rate of C/EBPa expression. This suggests that troglitazone influences factors that regulate when C/EBPα mRNA production is induced, but not those that modulate the total amount of C/EBPα mRNA expressed.

Troglitazone increases basal glucose uptake in differentiated adipocytes

To determine whether troglitazone has a direct effect on glucose metabolism in the adipocytes, the 3T3-L1 tissue culture system was used to mimic adipocyte function in a controlled environment. Initial experiments were performed using 3T3-L1 cells, which showed a 35% conversion frequency without troglitazone. Cells were differentiated under standard conditions with or without troglitazone for 8 days, as described in *Materials and Methods*. On day 9, basal glucose uptake was assessed by monitoring the accumulation of [^{14}C]deoxyglucose within the adipocytes. As shown in Fig. 3, 0.5 μM troglitazone treatment enhanced basal glucose uptake 9.6-fold in these cells. No effect was observed if cells were

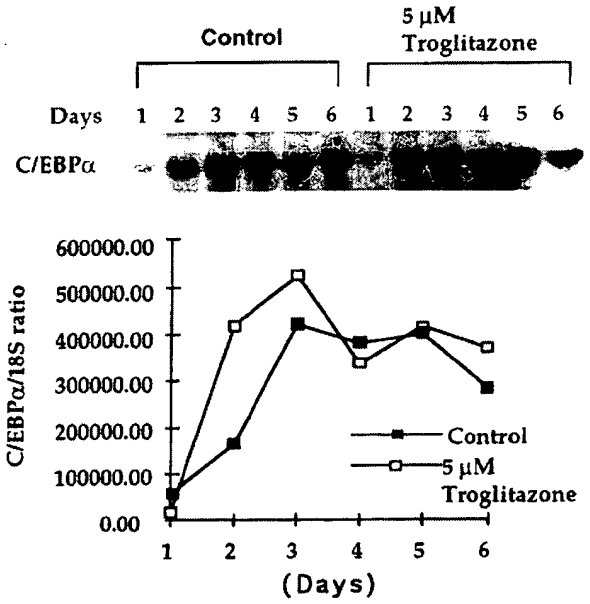

FIG. 2. Troglitazone affects the rate of C/EBPα message accumulation during differentiation, but not the overall level of message in the adipocyte. Northern analysis of total RNA collected every 24 h during adipocyte differentiation with and without 5 μM troglitazone using a ^{32}P-labeled, random primed, C/EBPα probe. RNA loading was evaluated by quantitation of 18S RNA. Multiple experiments demonstrated that the onset of C/EBPα expression varied with the passage number of the cells; however, in all cases, troglitazone enhanced the rate of C/EBPa expression.

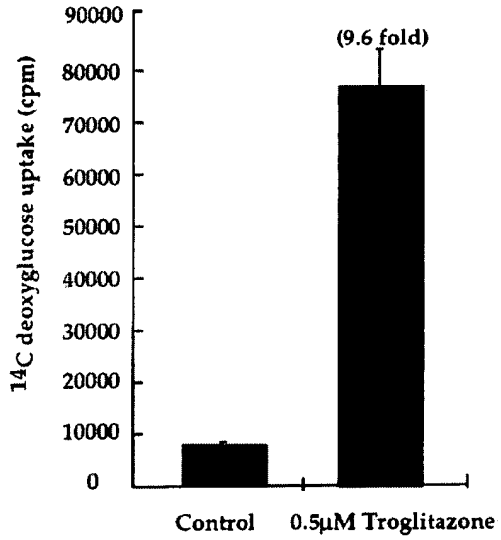

FIG. 3. Troglitazone treatment in combination with insulin increases basal glucose uptake in adipocytes. Adipocytes were differentiated using 100 nM insulin in combination with 0.25 μM dexamethasone and 0.5 mM isomethylbutylxanthine. After 8 days, the cell [^{14}C]deoxyglucose uptake was monitored, as described in *Materials and Methods*. The values were obtained from duplicate samples. When assayed, the control cells exhibited 10% differentiation, whereas the troglitazone-treated cells showed 80% differentiation.

treated without the addition of the hormone cocktail (data not shown). This suggests that troglitazone increases the activity or number of functional glucose transporters per cell, or both.

Noninsulin-dependent (basal) glucose uptake is a result of transport through the membrane-associated Glut1 glucose transporter, whereas insulin-stimulated glucose uptake results from the combined activities of Glut1 and the hormone-sensitive glucose transporter, Glut4, which are associated with both the plasma membrane and microsomal compartments. Comparison studies have shown that the levels of these two glucose transporter proteins differ between preadipocytes and adipocytes (13, 20, 21) (see Fig. 7). Total cellular levels of Glut1 decline slightly with differentiation. Conversely, the amount of Glut4 transporter in the adipocyte increases from undetectable in the preadipocyte to a value 2-fold greater than that of Glut1. Comparison of the adipocyte morphology between the cultures in the glucose uptake experiment in the previous experiment indicated that the troglitazone-treated samples had a higher adipocyte/preadipocyte ratio than the control samples (data not shown). Therefore, because the number and type of glucose transporters change during adipocyte differentiation, the adipocyte/preadipocyte ratio must be equivalent between samples to accurately determine how troglitazone affects glucose transport. To do this, we repeated the previous experiment using 3T3-L1 cells that differentiated more than 95% under standard differentiation conditions without troglitazone and whose morphology and final C/EBPα mRNA levels (Fig. 2) were not significantly enhanced by troglitazone treatment. In addition, we compared the basal glucose uptake to insulin-stimulated glucose uptake, which distinguishes between Glut1 and Glut4 transporter activities. As shown in Fig. 4,

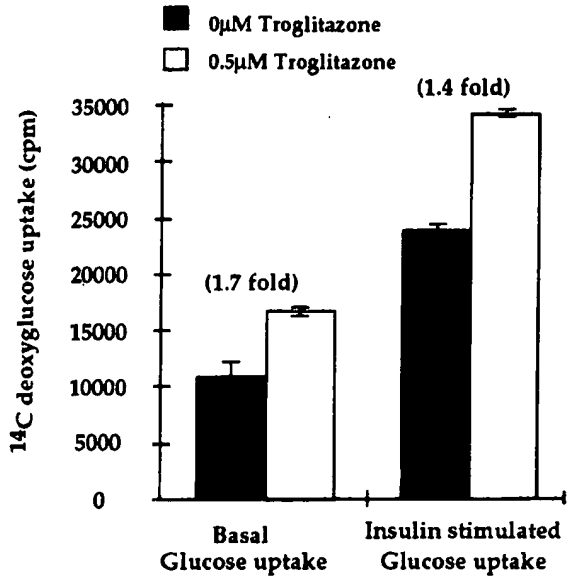

FIG. 4. Troglitazone increases basal glucose uptake in adipocytes. Adipocytes were differentiated under standard conditions in the presence or absence of 0.5 μM troglitazone. The percentage of adipocyte differentiation in all samples was more than 95%. [^{14}C]Deoxyglucose uptake was monitored 8 days after differentiation. For insulin-stimulated uptake, the cells were incubated for 30 min in 1×10^{-6} M insulin after a 3-h preincubation in serum-free DMEM. All values were obtained from duplicate samples.

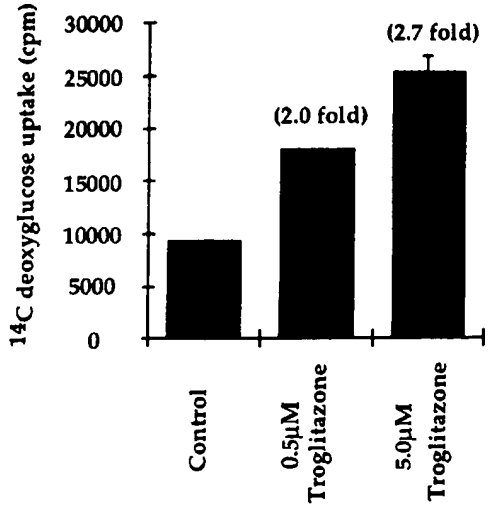

FIG. 5. Troglitazone enhances basal glucose uptake in fully differentiated adipocytes. Fully differentiated adipocytes were treated with 0, 0.5, or 5 μM troglitazone. Basal glucose uptake was assessed 48 h after treatment. All values were obtained from duplicate samples. The percentage of adipocyte differentiation in all samples was more than 95%.

basal glucose uptake increased 1.7-fold in response to 0.5 μM troglitazone. Similar results (1.4-fold) were found for Glut4-dependent insulin-stimulated glucose uptake. As basal glucose uptake is a function of Glut1 transporters, and insulin-stimulated uptake results from the combination of Glut1 and Glut4 transporter activities, the fact that troglitazone enhances both basal and insulin-stimulated glucose uptake equivalently suggests that the compound only alters Glut1 transporter activity.

To further separate the effects of troglitazone on glucose uptake from those on adipocyte differentiation, adipocytes differentiated in the absence of troglitazone were treated with 0.5 or 5 μM troglitazone for 48 h before the glucose uptake assay. As shown in Fig. 5, 0.5 and 5 μM troglitazone treatment produced 2- and 2.7-fold increases in basal glucose transport activity. As there was no change in morphology in the cells during treatment, these data show that troglitazone can enhance basal glucose uptake activity without affecting cell differentiation.

To determine whether the enhancement of glucose transporter activity is due to an increase in transporter number or an increase in transporter function, Western analysis was performed on whole cell lysates (from cells differentiated in the presence or absence of troglitazone) using either Glut1- or Glut4-specific antibodies (Fig. 6). Again, the adipocyte/preadipocyte ratio required to eliminate the effects of differentiation on transporter levels was identical in treated and untreated samples. As previously described (13, 20, 21), Glut1 transporter levels decreased slightly with adipocyte differentiation, whereas Glut4 levels increased dramatically. Troglitazone caused a 2.3-fold increase in Glut1 protein without altering Glut4 levels. Thus, troglitazone enhances glucose uptake by increasing the number of basal glucose transporters per cell. Additionally, a Glut1 RNase protection assay was performed on RNA isolated from cells differentiated in the presence or absence of 5 μM troglitazone (Fig. 7). The Glut1/actin ratio was approximately 1.5–2 fold higher in the RNA samples isolated from troglitazone-treated cells. This directly correlates with the 2-fold increase in glucose transport and shows that troglitazone increases the number of Glut1 transporters in adipocytes.

As differentiation also affects Glut1 mRNA stability, and a previous report (12) indicated that thiazolidinediones increased mRNA stability, a Glut1 RNase protection assay was performed on RNAs isolated from adipocytes treated with 5 μg/ml actinomycin D. Figure 8 shows that when the control cells and troglitazone cells displayed the same adipocyte/preadipocyte ratio, the rate of Glut1 mRNA decay was the same for both treated and untreated cells. Identical results were obtained from cells treated with pioglitazone, another antidiabetic thiazolidinedione (data not shown). Albeit indirectly, these data also suggest that the increase in Glut1 mRNA is due to an increase in Glut1 transcription.

Discussion

Troglitazone treatment of 3T3-L1 cells increases both the rate and percentage of adipocyte differentiation. This phenomenon is linked to the increased rate of C/EBPα accumulation in differentiating cells. Because this accumulation of C/EBPα message occurs within 24 h of drug treatment, it implies that troglitazone interacts with proteins that are present in the preadipocyte or are rapidly induced by the differentiating hormone cocktail. Recent work on adipocyte differentiation suggests that the proteins involved are members of the peroxisome proliferator-activated receptor (PPAR) family (22, 23). These nuclear receptors are activated by endogenous fatty acid or PG ligands (24–26) and in combination with C/EBP family members are believed to induce

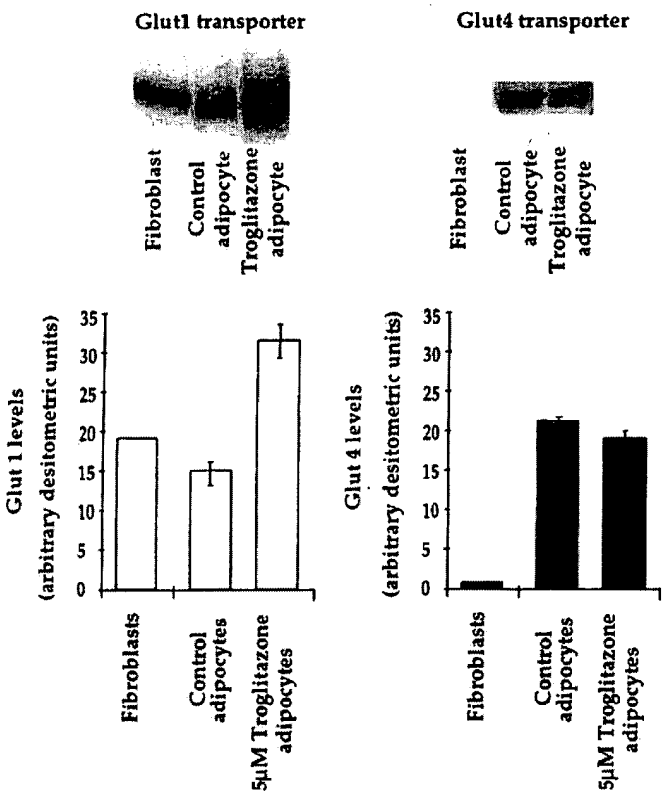

FIG. 6. Troglitazone increases the synthesis of the Glut1 transporter protein in adipocytes. Western analysis was performed using a Glut1- or Glut4-specific antibody of protein samples collected from adipocytes treated with or without 5 μM troglitazone. The percentage of adipocyte differentiation in all samples was more than 95%. Thirty micrograms of protein were loaded per lane, as determined using Bio-Rad analysis. Histograms represent data acquired from two independent experiments.

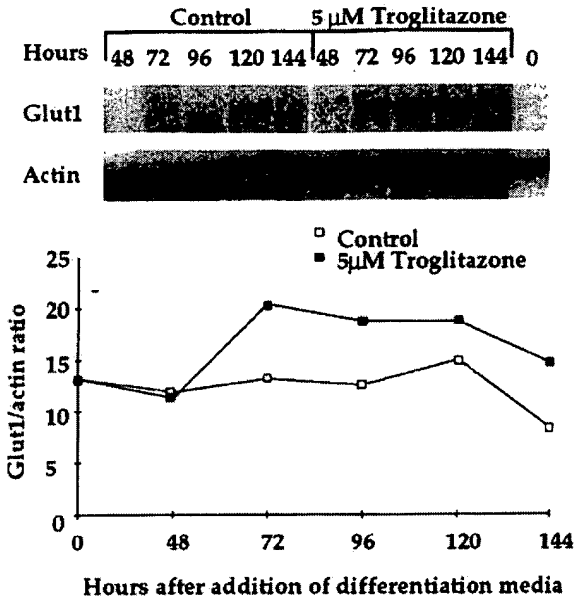

FIG. 7. The levels of Glut1 mRNA were increased in response to 5 μM troglitazone. A RNase protection assay was performed on RNAs collected at the indicated times from cells treated with or without 5 μM troglitazone. Ten micrograms of RNA per sample were hybridized to mouse Glut1 and β-actin probes and processed as described in *Materials and Methods*. Message levels were quantitated using a Molecular Dynamics PhosphorImager, and the values are represented as the Glut1/actin ratio.

adipocyte differentiation (27–29). Lehmann and colleagues (30) have shown that another thiazolidinedione, BRL 49653, is a ligand for PPARγ, the adipocyte-specific PPAR family member. This suggests that by interacting with the PPARs, troglitazone initiates the cascade of transcriptional events that enhances the rate of adipogenesis.

To determine whether the increased glucose uptake in adipocytes *in vivo* is a direct effect of troglitazone or a consequence of secondary effects brought about by insulin sensitization, the effects of troglitazone were assessed in the isolated 3T3-L1 tissue culture system. Apart from the enhanced glucose uptake associated with adipocyte differentiation, troglitazone directly increased glucose uptake 2-fold in these cells. This effect was shown to be a direct result of an increased synthesis of Glut1 transporter mRNA and protein. This disagrees with previously reported results (12), which have shown that pioglitazone, another thiazolidinedione with antidiabetic activity, enhances glucose uptake in 3T3-442A adipocytes by increasing both Glut1 and Glut4 transporter mRNA and protein via mRNA stabilization. However, this study failed to distinguish between the increases in glucose uptake associated with the enhancement of adipocyte differentiation and those resulting from the direct effects of the thiazolidinedione on the adipocyte. By controlling for the ratio of adipocytes/preadipocytes in the experiments presented here, the indirect effects of differentiation by the compound on Glut4 were eliminated, demonstrating that troglitazone enhances glucose uptake by increasing Glut1 mRNA and protein levels. Additionally, identical experiments with pioglitazone yielded similar results (data not shown).

These results provoke at least two questions. First, can a 2-fold increase in adipocyte glucose uptake account for the decreased hyperglycemia *in vivo* or must other tissues also be affected? Secondly, are the transcriptional responses involved in differentiation the same as those used in the expression of Glut1; do both require the activation of PPARs by the thiazolidinediones?

Glucose transporter number has been shown to directly affect glucose transport and blood glucose levels in animal models. Several transgenic mice have been engineered that overexpress the glucose transporters in a tissue-specific fashion (30–34). In general, overexpression of either Glut1 or Glut4 enhanced glucose transport in the targeted tissue. Enhanced transport directly correlated with decreased plasma glucose levels in both fasted and fed animals, demonstrating that enhanced transporter expression has profound effects on glucose disposal *in vivo*. Surprisingly, however, increased Glut1 expression in skeletal muscle, and hence increased muscle basal transport, resulted in resistance of Glut4 to insulin stimulation and various other stimuli, including contraction and hypoxia (32). Additionally, Glut 4 overproduction in fat cells did not protect animals from the impaired glucose tolerance induced by a high fat diet (34). Thus, although enhanced transporter synthesis can ameliorate hy-

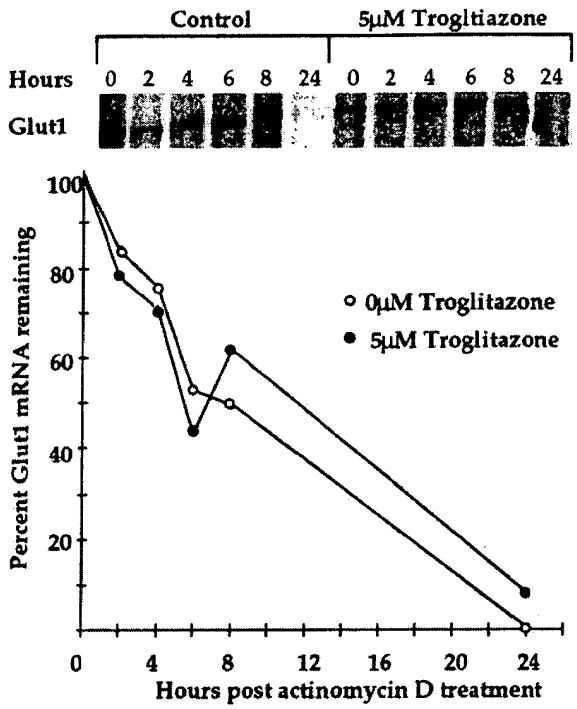

FIG. 8. Troglitazone does not alter the stability of Glut1 mRNA. RNase protection assay of Glut1 message was performed on RNA collected after actinomycin D (5 μg/ml) treatment of adipocytes differentiated in the presence or absence of 5 μM troglitazone. Ten micrograms of RNA were used per sample, and message levels were quantitated using a Molecular Dynamics PhosphorImager.

perglycemia, it does not appear to directly influence insulin resistance. This would imply that enhanced Glut1 synthesis in adipocytes would only represent a portion of troglitazone's antidiabetic activity. This is supported by data indicating that troglitazone influences the metabolic activities of skeletal muscle, liver, and pancreas (35–38).

The precise role of PPARs in the antidiabetic activities of the thiazolidinediones has yet to be fully explored. Clearly, the PPARs are intimately involved in lipid metabolism in a number of tissues, including adipose and liver, and are responsible for the lipid-lowering effects of the fibric acids (39). Interestingly, the lipid-lowering effects associated with thiazolidinedione treatment are similar to those of other PPAR-activating, lipid-lowering compounds (40). Moreover, as elevated lipid levels have been linked to peripheral insulin resistance, the alteration of lipid metabolism may ameliorate insulin resistance (41). However, *in vitro*, thiazolidinediones bind specifically to the PPARγ isoform, which is mainly present in adipose tissue (22, 23). Thiazolidinediones have been shown to influence metabolism in liver, pancreas, and skeletal muscle. If all of these antidiabetic responses are to be attributed to PPAR activation, all insulin-responsive tissues must contain sufficient levels of this isoform. Alternatively, it is possible that the ligand binding specificity *in vivo* differs from that *in vitro*, and/or that the compounds stimulate the formation of ligands that activate the PPAR isoforms present in these other tissues. Future studies addressing these issues will undoubtedly reveal the role of the PPARs in the antidiabetic activity of the thiazolidinediones. However, it is clear that the regulation of Glut1 transporter synthesis could contribute to the antidiabetic activity of troglitazone.

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The Antidiabetic Agent Pioglitazone Increases Expression of Glucose Transporters in 3T3-F442A Cells by Increasing Messenger Ribonucleic Acid Transcript Stability*

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ABSTRACT

Whereas adipocytes normally play an important role as a major site for systemic energy homeostasis, adipocyte function is markedly altered in disorders such as diabetes. In this study, we investigated the effect of pioglitazone, a novel antidiabetic agent known to lower plasma glucose in animal models of diabetes mellitus, on expression of glucose transporters GLUT1 and GLUT4 in 3T3-F442A cells. Treatment of confluent 3T3-F442A preadipocyte cultures for 7 days with pioglitazone (1 μM) and insulin (1 μg/ml) resulted in nearly 100% differentiation of cells to lipid-accumulating adipocytes, and such adipocytes showed a markedly increased capacity for glucose uptake. Analysis of messenger RNA transcripts encoding GLUT1 and GLUT4 glucose transporters over the 7-day differentiation period indicated time-dependent increases in abundance of each type that were maximal at more than 5-fold with the combined presence of insulin and pioglitazone. In accord, GLUT1 and GLUT4 protein levels also increased to maximal levels of 10-fold and 7-fold, respectively, over those in undifferentiated preadipocytes. Increased messenger RNA half-lives from 2.2 to greater than 24 h for GLUT1 and from 1.2 to greater than 24 h for GLUT4 correlated with this induced adipocyte differentiation. Taken together, these findings indicated that pioglitazone markedly enhanced expression of cellular glucose transporters, and the mechanism for this action was mainly stabilization of transporter messenger RNA transcripts. Such increased expression of glucose transporters in adipocytes establishes the cells in a state active for glucose uptake, thus ultimately facilitating storage and metabolism as well. (*Endocrinology* **133:** 352–359, 1993)

ADIPOSE tissues play a key role in systemic energy homeostasis. Adipocytes possess hormonally regulated transport and metabolic systems allowing energy storage as triglycerides when nutrients abound or energy release during nutritional dearth. In accord with this role, a recent report indicated that adipose may be responsible for up to 30% of whole body glucose metabolism (1). Altered adipocyte function has been associated with abnormal physiological states including obesities and obesity-linked diabetes (2–4). In non-insulin-dependent diabetes mellitus, elevated blood glucose levels result from insufficient glucose uptake in adipose and muscle, a consequence of insulin resistance (5, 6).

The hyperglycemia of noninsulin-dependent diabetes mellitus can be corrected clinically by treatment of patients with oral hypoglycemic agents. Whereas presently used sulfonylurea agents appear to act principally as secretagogues to increase the availability of insulin to enhance glucose disposal (7, 8), an alternate treatment strategy could employ agents acting as insulin sensitizers, thus overcoming target tissue insulin resistance. New antidiabetic compounds belonging to the thiazolidinedione class of drugs appear to lower blood glucose in animal models of diabetes by improving insulin sensitivity in peripheral tissues (9). Treatment of insulin-resistant fatty rats or mice with the thiazolidinedione pioglitazone resulted in lowered blood glucose, triglyceride, and insulin levels (10, 11).

We and others have previously reported that thiazolidinedione agents enhanced insulin sensitivity for glucose uptake and metabolism in adipose tissues of diabetic animals (10–13). It has also been found that such agents have potent adipogenic effects on preadipocyte cell cultures (14–16). We therefore sought to more fully investigate the mechanism underlying the effect of pioglitazone to promote adipocyte differentiation with the aim of gaining insight into how such an effect could contribute to regulation of cellular glucose uptake. Findings in our present report indicate that pioglitazone treatment of 3T3-F442A preadipocytes markedly enhances expression of glucose transporters GLUT1 and GLUT4, and the mechanism for this action is a stabilization of transporter messenger RNA (mRNA) transcripts.

Materials and Methods

Cells and tissue culture

3T3-F442A fibroblasts were grown as monolayer cultures at 37 C in an atmosphere of 10% CO_2-90% air essentially as described previously (17). Subcultured cells were grown to confluence (usually 7 days) in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose (4.5 g/L), bovine serum (10%), streptomycin (50 μg/ml), penicillin (50 U/ml), Fungizone (0.25 μg/ml), and glutamine (2 mM). Confluent cell cultures were then converted to adipocytes by culture for 7 days in

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DMEM in the presence of 10% fetal calf serum and insulin (1 μg/ml) and/or pioglitazone (1 μM). Cell differentiation was assessed by evaluating cell morphology under phase contrast microscopy; cells were considered to be adipocytes if numerous lipid droplets were observed in the cytoplasm. The cell line was used during the 10th to 20th passage after clone isolation.

*Measurement of 2-deoxy-*D[^{14}C]glucose uptake

For assay, 3T3-F442A cell monolayers (~10^6 cells/17 mm well) were rinsed with PBS and incubated with 0.5 ml assay medium (DMEM, no glucose, 5 mM $NaHCO_3$, 20 mM *N*-[2-hydroxyethyl]-*N'*piperazine-[2-ethanesulfonic acid], 0.1% BSA, pH 7.4) for 15 min at 22 C. Then 0.5 μCi D-[U-^{14}C]glucose (stock = 57 mCi/mmol, Amersham Corp., Arlington Heights, IL) was added for an additional 15 min. After this incubation, medium was aspirated, and cells were rinsed with ice-cold PBS containing 10 mM glucose. Cells were then solubilized with 0.5 ml 0.5 N NaOH, transferred to vials, neutralized with 52 μl glacial acetic acid, and counted for radioactivity using 5 ml Ready Value scintillation fluid (Beckman, Inc., Palo Alto, CA).

Northern blot analysis of RNA for determination of GLUT1 and GLUT4 mRNA transcript abundance

Total RNA was extracted from 3T3-F442A cell monolayers essentially according to the method of Chomczynski and Sacchi (18). RNA was then size-fractionated on 1% agarose gels and transferred to nylon membranes according to the method of Fourney *et al.* (19). RNA transcripts were cross-linked to the membrane with a UV Stratalinker (Stratagene, La Jolla, CA).

Hybridization was performed using anti-sense RNA Riboprobes prepared according to the protocol of the reagent supplier (Promega Corp., Madison, WI). For GLUT1, pSPGT-1 (20) was kindly provided by Dr. Graeme Bell (Chicago, IL), and the entire 1591 base pair coding region of rat brain glucose transporter was excised using *Bgl*II and subcloned into the *Bam*HI site of pGEM-4Z. *Eco*RI was used for linearization, and T7 RNA polymerase was used for GLUT1 Riboprobe transcription. A GLUT4 construct pSM1D2 in the pBluescript KS$^+$ (21) was generously provided by Dr. Morris Birnbaum (Boston, MA). For GLUT4 Riboprobe preparation, this construct was linearized with Hind III, and T7 RNA polymerase was used for Riboprobe preparation.

High stringency hybridizations with Riboprobes were performed by 65 C overnight incubation of membranes in glass tubes in a Hybaid Oven (National Labnet Co., Woodbridge, NJ) using procedures we have detailed previously (11). After rinses, labeled membranes were exposed at −70 C to Hyperfilm-MP (Amersham Corp., Arlington Heights, IL) with an intensifying screen. Autoradiographic bands on film were quantitated by two-dimensional densitometry using an AMBIS Optical Imaging System (San Diego, CA). RNA samples were quantitated for correction of minor loading differences by densitometry of ethidium bromide-stained 28S ribosomal bands (22) on photographic negatives (type 55 P/N film, Polaroid, Cambridge, MA).

Measurement of GLUT1 and GLUT4 mRNA stability

Confluent 3T3-F442A cells were induced to differentiate with insulin, pioglitazone, or both as described above. On day 7 of treatment, the transcription inhibiting agent Actinomycin D (Act D) was added (5 μg/ml) to treated cell cultures as well as undifferentiated control cultures, essentially as reported earlier (23–26). Total RNA was extracted from these cells at selected time points after Act D addition (0, 1, 2, 4, 6, and 24 h), and abundance of remaining GLUT1 and GLUT4 mRNA transcripts was assessed by Northern blot analyses as described above. Glucose transporter mRNA abundance data were fitted to a single exponential decay curve by nonlinear least square regression analysis. The estimated first-order rate constant was used to calculate the mRNA half-life. Since it was suggested that Act D may have nonspecific effects with long-term (24 h) treatments (24), glucose transporter mRNA half-life calculations were based on changes in mRNA abundance over only the first 6 h of Act D treatment.

Western blot analysis of glucose transporter proteins

Total particulate membrane proteins were prepared as previously described (27). Briefly, cells were washed with PBS and scraped into homogenization buffer [20 mM Tris-HCl, 255 mM sucrose, 1 mM EDTA, 1 mM phenylmethyl sulfonylfluoride, 10 U/ml Trasylol] and were homogenized with 10 pulses by a Tekmar Tissuemizer (Tekmar Inc., Cincinnati, OH). A total membrane fraction was prepared by centrifugation of the homogenate at 200,000 × *g* at 4 C. Protein concentrations were determined by the Bradford assay (28) using BSA as a standard. Initial Western blot analyses revealed diffuse bands of GLUT1 and GLUT4 proteins, possibly due to heterogenous glycosylation of the transport proteins. Therefore, samples were routinely treated with peptide *N*-glycosidase F (1 U/100 μg protein) in a buffer containing 20 mM sodium phosphate, pH 7.5, 10 mM EDTA, 1.7% Triton X-100, and 1 mM phenylmethyl sulfonylfluoride for 48–72 h at 37 C to remove sugar residues (23). Treated protein samples were then mixed with one fourth volume of 4× electrophoretic sample buffer [200 mM Tris-HCl at pH 6.8, 400 mM dithiothreitol, 8% sodium dodecyl sulfate, 40% glycerol, 0.4% bromophenol blue] and stored at −20 C. Samples were thawed and loaded in parallel onto two discontinuous 12% polyacrylamide gels, and size-fractionated according to the method of Laemmli (29) using a Mini-Protean II Dual Slab Cell (Bio-Rad, Richmond, CA). The amount of protein loaded (10 μg/lane for GLUT1 and 60 μg/lane for GLUT4) was determined empirically to be within the linear response range for the system used. Proteins separated on each gel were electrophoretically transferred to Immobilon PVDF membranes (Millipore Corp., Bedford, MA) using a Mini-Transblot electrophoretic transfer cell (Bio-Rad). One membrane was stained for total protein (0.1% Coomassie R-250, 40% methanol, 10% acetic acid). The second membrane was immunostained for GLUT1 or GLUT4 using a double antibody system and the Immuno-Blot Assay Kit (Bio-Rad). The supplier's instructions were followed except 5% BSA was used for membrane blocking. The primary antibodies (RaGLUTRANS for GLUT1 and RaIRGT for GLUT4, East Acres Biologicals, Southbridge, MA) were diluted 1:3000 and 1:2000, respectively, and the alkaline phosphatase-conjugated second antibody (GAR-AP, Bio-Rad) was diluted 1:3000 for use. Resulting signals were quantitated with the reflective mode of a Model 620 Video Densitometer (Bio-Rad). Sample loading corrections were made based on densitometry data from the Coomassie-stained membrane.

Data analysis

Statistical analysis was performed using SAS version 6 (SAS Institute, Inc., Cary, NC). All hypothesis tests were two-sided and were considered significant if the *P* value was less than or equal to 0.05.

Results

Induction of preadipocyte differentiation and glucose transport by insulin and pioglitazone

Treatment of confluent 3T3-F442A cells for 7 days with insulin (1 μg/ml), pioglitzone (1 μM), or both agents in the presence of 10% fetal calf serum resulted in conversion of cells into lipid-accumulating adipocytes. Whereas some cells differentiated into adipocytes by either treatment alone (insulin, 60%; pioglitazone, 80%), nearly complete differentiation (95%) was achieved with both agents together. Such cellular differentiation was associated with markedly increased capacity for glucose transport. Treatment of 3T3-F442A cells for 7 days with insulin or pioglitazone resulted in basal glucose transport levels that were 22- and 30-fold increased compared to untreated fibroblasts (Table 1). Together, both agents appeared to act additively for a maximal enhancement of 61-fold at day 7. Age-matched, undifferentiated cells maintained in growth medium showed no

TABLE 1. P and I-induced increases in glucose transport activity in differentiating 3T3-F442a Cells

Cell treatment	Glucose uptake (dpm/cell ± SEM)
Control, day 0	0.0030 ± 0.00007
Control, day 7	0.0013 ± 0.00031
Insulin	0.0670 ± 0.0076
Pioglitazone	0.0887 ± 0.015
Insulin + pioglitazone	0.1830 ± 0.037

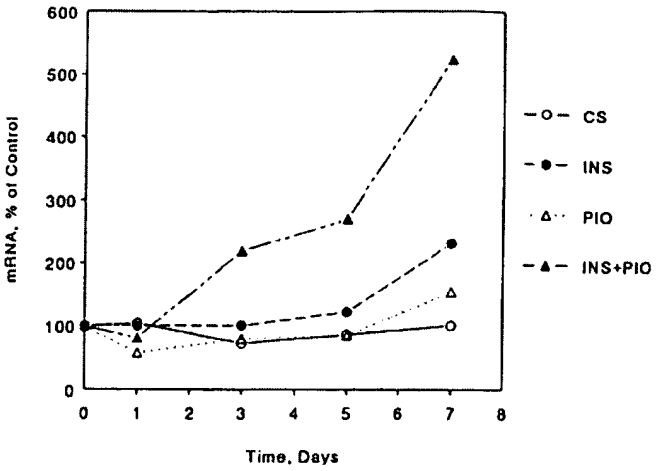
Confluent monolayer cultures of preadipocytes in DMEM (day 0) were induced to differentiate by treatment with insulin and/or pioglitazone. Preadipocyte cells maintained for 7 days in DMEM without added insulin and/or pioglitazone also served as controls. After treatment for 7 days, glucose transport actvity was measured by uptake of 2-deoxy-D[^{14}C]glucose as described in *Materials and Methods*. Since the radiolabeled glucose has a specific activity of 300 mCi/mmol, there are 1.5 f/dpm. Thus, maximal basal transport was approximately 0.3 fmol/cell for cells differentiated in medium containing insulin and pioglitazone.

increase in glucose transport over the 7-day interval, and actually declined about 60%, likely an indication of quiescence.

Insulin- and pioglitazone-enhanced expression of glucose transporters in 3T3-F442A cells

To assess whether such increased glucose transport activity could be explained by amplified expression of glucose transporters in adipocytes, we analyzed the levels of GLUT1 and GLUT4 mRNA abundance. When assessed by Northern blotting, the abundance of mRNA transcripts encoding GLUT1 and GLUT4 glucose transporters increased in a time-dependent manner (Figs. 1, 2). Whereas 7-day treatment with either insulin (1 μg/ml) or pioglitazone (1 μM) increased GLUT1 mRNA abundance by 2.3- and 1.5-fold, respectively, above the level in undifferentiated cells (day 0), insulin and pioglitazone together acted synergistically to increase this message by almost 6-fold (Fig. 1). GLUT1 mRNA levels did not change in age-matched undifferentiated cells maintained in growth medium over the same interval. In contrast to the observed synergistic treatment effects on GLUT1 mRNA levels, GLUT4 mRNA abundance was increased to similar levels above undifferentiated cells (day 0) reaching 3.8-, 4.6-, and 5.2-fold elevation by respective treatments for 7 days with insulin, pioglitazone, or both (Fig. 2). Whereas these values for GLUT4 mRNA abundance reflected an overall increase at day 7, a small decline was observed at this time point in some experiments with combined treatment by insulin and pioglitazone (Fig. 2, lower). Such results indicate that some down-regulation may occur in the final differentiated state.

These changes in GLUT1 and GLUT4 glucose transporter mRNA levels were accompanied by changes in levels of the encoded transporter proteins as determined by Western blotting. Whereas either insulin or pioglitazone treatment appeared to increase GLUT1 protein levels on day 7 of differentiation by 2.3- and 3.5-fold respectively, the agents together seemed to act synergistically to increase protein levels by almost 10-fold above those of age-matched undifferentiated cells (Fig. 3). In contrast to the observed synergistic



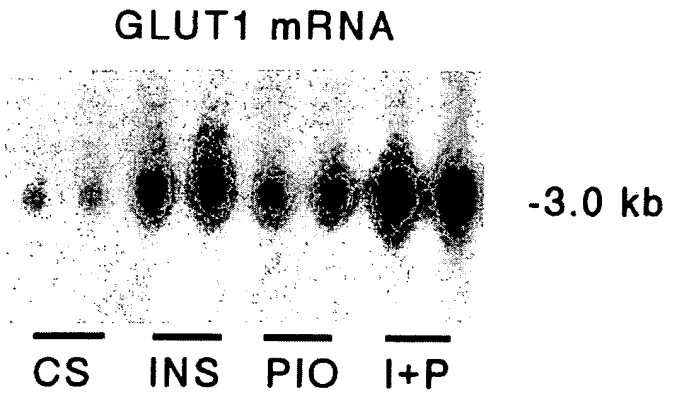


FIG. 1. Effect of insulin and pioglitazone treatment on GLUT1 mRNA abundance. *Upper*, Time-dependent increases in abundance of GLUT1 mRNA during differentiation of 3T3-F442A cells induced by insulin (INS), pioglitazone (PIO), or both. Cells were seeded, grown to confluence, and differentiated as described in *Materials and Methods*. Untreated age-matched fibroblasts maintained in DMEM containing only 10% calf serum were used as control (CS). Total RNA was isolated at indicated time points, and samples (10 μg/lane) were electrophoretically size-fractionated on agarose gels. Northern blots were hybridized to Riboprobes specific for rat GLUT1 mRNA. Autoradiographic bands were quantitated by densitometry and normalized for minor loading differences as described in *Materials and Methods*. Data represent mean values for n = 5–6 determinations. A three factor analysis of variance (ANOVA; insulin, pioglitazone, time) with interactions showed a significant effect on GLUT1 mRNA abundance from combined treatment with I and P ($P < 0.001$). *Lower*, Representative Northern blot showing GLUT1 mRNA abundance in control (CS) and treated (INS, PIO, I + P) cells on day 7 of differentiation.

effect on GLUT1 protein, GLUT4 protein levels increased by about 2.2-, 6.6-, and 4-fold by insulin, pioglitazone, or both, respectively (Fig. 4).

Increased stability of glucose transporter messages

We next investigated possible effects of insulin and pioglitazone on the stability of GLUT1 and GLUT4 mRNAs. Fibroblast 3T3-F442A cells were induced to differentiate by treatment with insulin (1 μg/ml), pioglitazone (1 μM), or both for 7 days. The transcription inhibiting agent, Act D (5 μg/ml), was added to differentiated cells on day 7 or to undifferentiated control cells just before such cells reached conflu-

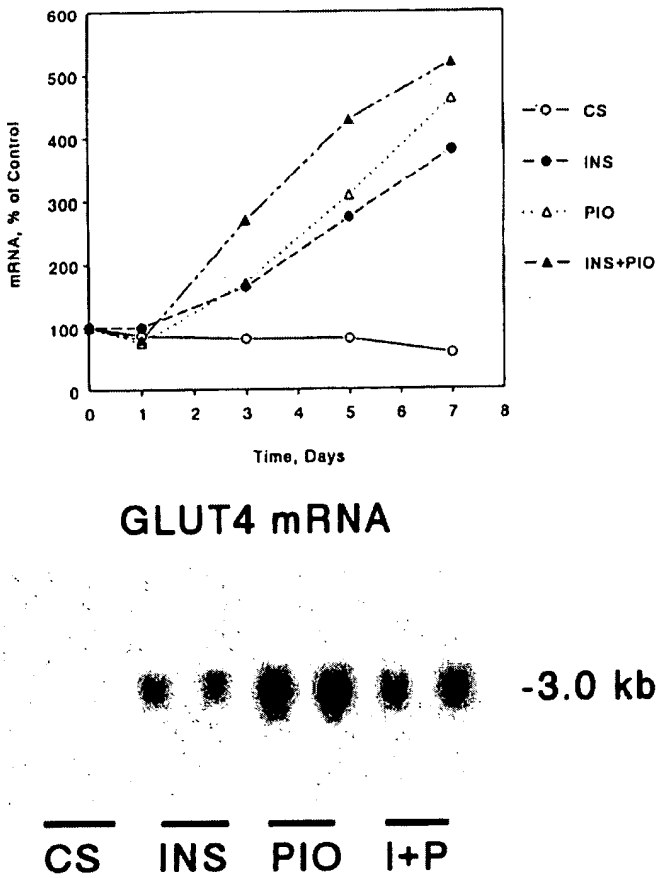


FIG. 2. Effect of insulin and pioglitazone treatment on GLUT4 mRNA abundance. *Upper*, Time-dependent increases in GLUT4 mRNA abundance in 3T3-F442A cells during differentiation. Cells were grown to confluence, and differentiated in the presence of insulin (INS), pioglitazone (PIO), or both (INS + PIO) as described in *Materials and Methods*. Untreated, age-matched fibroblasts maintained in DMEM containing only 10% calf serum were used as control (CS). Total RNA was isolated, Northern blots were prepared as described for Fig. 1, and membranes were hybridized with Riboprobe transcripts specific for rat GLUT4. Quantitation of resultant autoradiographs was done as described in *Materials and Methods*. Data represent mean values for n = 5–6 determinations. A three factor ANOVA (insulin, pioglitazone, time) with interactions showed significant effects on GLUT4 mRNA abundance by INS ($P = 0.001$) or PIO ($P < 0.0001$). *Lower*, Northern blot showing GLUT4 mRNA abundance in control (CS) and treated (INS, PIO, I + P) cells on day 7 of differentiation.

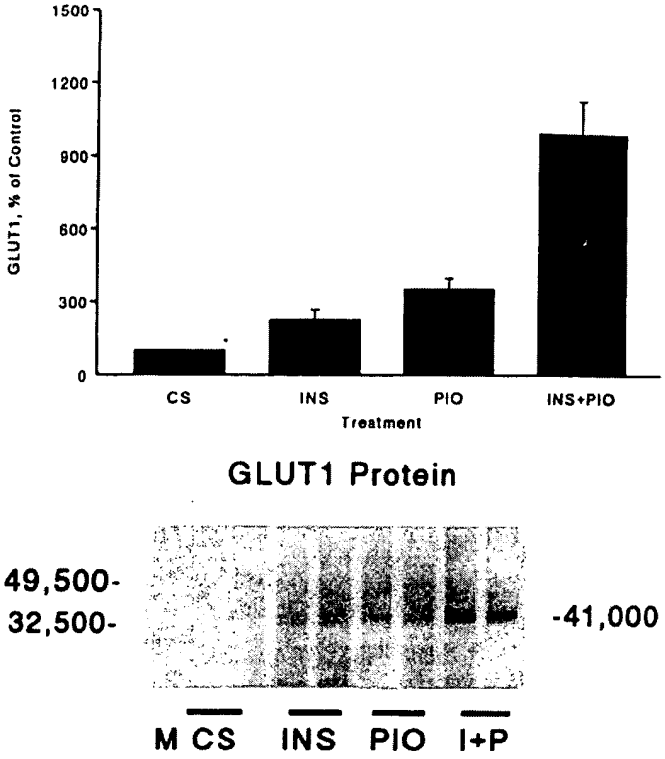


FIG. 3. Effect of insulin and pioglitazone treatments on GLUT1 protein abundance. *Upper*, Increased GLUT1 protein abundance on differentiation of 3T3-F442A cells. Confluent 3T3-F442A cells were differentiated with I (INS), P (PIO), or both (INS + PIO) as described in *Materials and Methods*. On day 7, cellular membranes were isolated from undifferentiated (CS) or differentiated (INS-, PIO-, or INS + PIO-treated) cells, and protein (10 μg/lane) was electrophoresed and immunoblotted for GLUT1 detection using the rabbit anti-GLUT1 primary antibody (see *Materials and Methods*). Resulting colorimetrically stained bands were quantitated by densitometry and normalized for minor loading differences. Data are expressed as percent of control and represent mean values ± SEM for n = 8 determinations. With background subtraction, the mean basal value was 0.20 ± 0.06 arbitrary density units. Two factor ANOVA showed significant effects on GLUT1 protein by INS ($P = 0.014$), PIO ($P = 0.015$), and INS + PIO ($P < 0.0001$). *Lower*, Representative immunoblot showing expression of GLUT1 protein in selected samples from control (CS) and treated (INS, PIO, I + P) cells on day 7 of differentiation. Molecular weight markers are indicated in lane M; the GLUT1 band ran as $M_r = 41,000$.

ence, and total RNA was extracted from cells at indicated times after addition of the transcription inhibitor (0, 1, 2, 4, 6, and 24 h). Even after 24-h treatment with Act D, cell membranes remained intact and appeared refractile, and there was no visible sloughing of cells from the plates. The abundance of remaining mRNA transcripts for each of the glucose transporter genes was assessed on Northern blots. Differentiation of 3T3-F442A cells increased the GLUT1 mRNA half-life from about 2.2 h in control undifferentiated cells to about 5.7, 3.6, and greater than 24 h in adipocytes differentiated by treatment with insulin, pioglitazone, or both, respectively (Fig. 5, Table 2). The stabilization of GLUT1 mRNA appeared to account for corresponding increases in GLUT1 mRNA abundance. Similarly, GLUT4 mRNA half-life increased from about 1.2 h in undifferentiated control cells to about 14.2, 10.3, and greater than 24 h in cells differentiated with insulin, pioglitazone, or both, respectively (Fig. 5, Table 2). Again, GLUT4 mRNA stabilization appeared to account for observed increases in mRNA abundance.

As shown in Fig. 6, we were able to quantitate differential expression of transporter messages in cells undergoing different treatments by differentially exposing the Northern blots to film. This allowed calculation of message half-lives even when transcripts were present only at relatively low levels.

Discussion

Pioglitazone, 5-[4-(2-(5-ethyl-pyridyl)ethoxy]-2,4-thiazolidinedione, is an antidiabetic agent that has been shown to

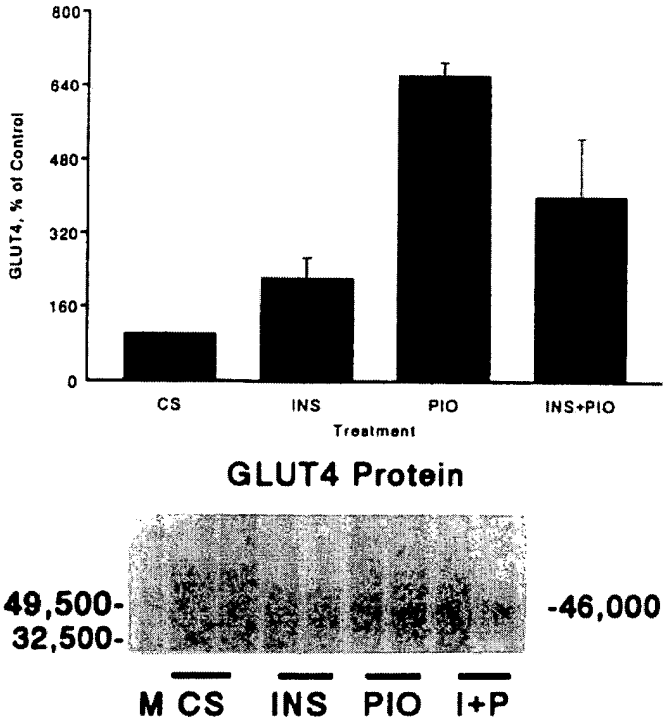


FIG. 4. Effect of insulin and pioglitazone treatments on GLUT4 protein abundance. *Upper*, Increased GLUT4 protein levels on differentiation of 3T3-F442A cells. Cell culture, treatment conditions, protein extraction, and Western blot analyses were performed as described in Fig. 3 legend, except that 60 μg/lane total protein were loaded for detection using the rabbit anti-GLUT4 primary antibody. Data are expressed as percent of control and represent mean ± SEM for n = 10 determinations. After background subtraction, the mean basal value was 0.57 ± 0.11 arbitrary density units. Two factor ANOVA showed significant effects on GLUT4 by INS ($P = 0.015$), PIO ($P = 0.007$), and INS + PIO ($P = 0.0003$). *Lower*, Representative immunoblot showing increased levels of GLUT4 protein in 7 day-treated (INS, PIO, I + P) compared to untreated control (CS) cells. GLUT4 protein appears as a stained band with apparent M, of 46,000. In control cells (CS), a nonspecific band of apparent M, 60,000 cross-reacted with the secondary antibody.

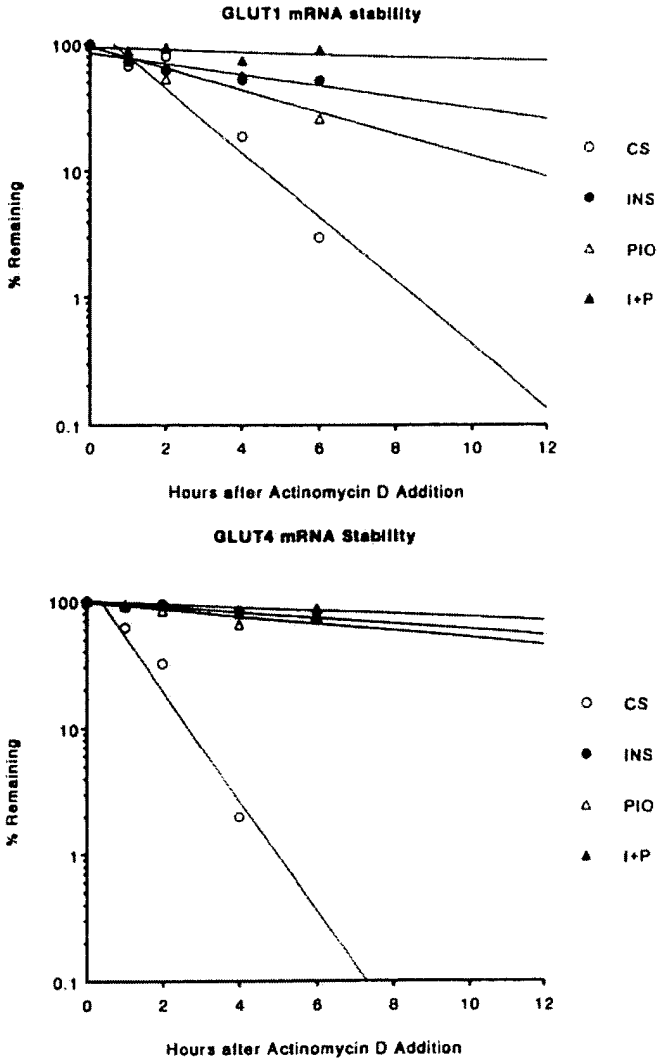


FIG. 5. *Upper*, Enhanced GLUT1 mRNA stability associated with treatment of 3T3-F442A cells with insulin (INS), pioglitazone (PIO), or both (I + P). *Lower*, Enhanced GLUT4 mRNA stability associated with treatment of 3T3-F442A cells with I, P, or both. Confluent 3T3-F442A cells were differentiated with INS, PIO, or I + P for 7 days as described in *Materials and Methods*. On day 7 of treatment, Act D (5 μg/ml) was added to differentiated (INS, PIO, I + P) and undifferentiated (CS) control cells, and total RNA was extracted from cells at indicated time points (0, 1, 2, 4, 6, and 24 h). Abundance of mRNA was assessed by Northern blotting analysis as described earlier. Data are expressed as percent of mRNA remaining after Act D treatment relative to the levels before the treatment time 0. Each data point represents a mean value for n = 2 determinations. Calculated mRNA half-lives are shown in Table 2.

ameliorate hyperglycemia in animal models of noninsulin-dependent diabetes mellitus (10, 11, 30). The purpose of our study was to further probe cellular action mechanisms underlying the antidiabetic effects of pioglitazone. Our prior findings indicated that the antidiabetic agent pioglitazone acted as a potent accelerator of adipocyte differentiation of 3T3-F442A cells (14). This was evidenced by our demonstration that treatment of fibroblast-like preadipocytes with pioglitazone led to expression of fat-specific genes along with acquisition of the morphological appearance of lipid-accumulating adipocytes with concomitant increases in triglyceride accumulation (14). In the present study, we showed that such pioglitazone-treated cells showed an increased capacity for glucose uptake, with associated increases in GLUT1 and GLUT4 proteins. We further measured increased levels of mRNA transcripts encoding these glucose transporters, and found that these increases corresponded with markedly enhanced stability of both GLUT1 and GLUT4 mRNA messages. Such increased expression of glucose transporters in adipocytes established the cells in a state active for glucose uptake, thus ultimately facilitating glucose storage and metabolism as well.

Results of our present study showed that the differentiation of 3T3-F442A cells by treatment with insulin and pioglitazone was accompanied by strikingly increased capacity for basal glucose transport (60-fold) compared to that for fibroblast-like preadipocytes. Facilitated diffusion of glucose across the plasma membrane of adipocytes is known to be mediated by two glucose transporter proteins, *i.e.* GLUT1

TABLE 2. Calculated mRNA half-lives in undifferentiated and differentiated 3T3-F442A cells

Treatment	mRNA half-life (h)			
	CS	INS	PIO	INS + PIO
Transporter				
GLUT1	2.2	5.7	3.6	>24
GLUT4	1.2	14.3	10.3	>24

Data from the experiment involving Act D treatment (Fig. 5) were fitted to a single exponential decay curve by nonlinear least square regression analysis. The estimated first-order rate constant was used to calculate the mRNA half-life.

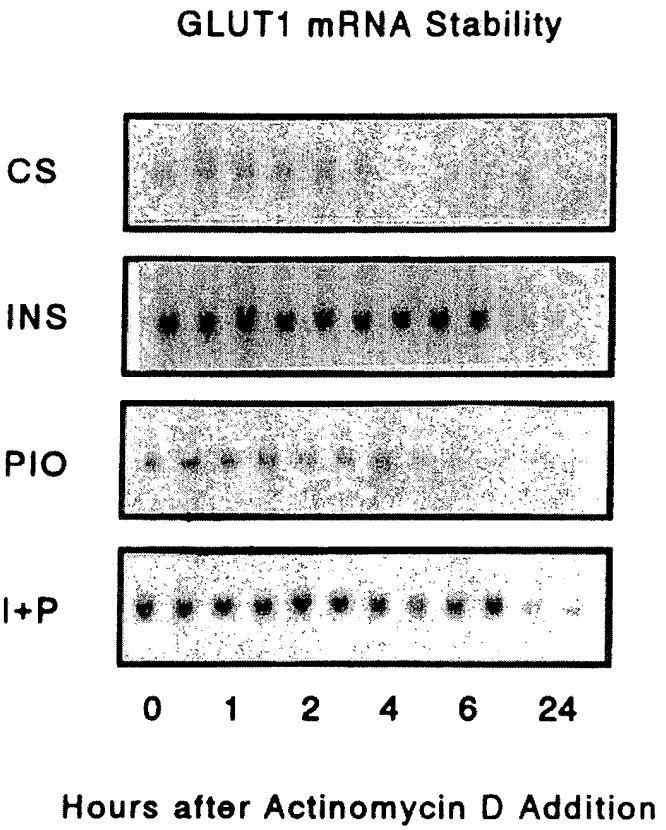


FIG. 6. Autoradiographs of Northern blots probed for GLUT1 mRNA transcript abundance after D treatment. Experimental samples were prepared as described in Fig. 5. Data shown represent duplicate experimental points, except for the time 0 INS, where only a single sample is shown. Hours of film exposure to the radiolabeled blot were 40, 18, 24, and 8 for CS, INS, PIO, and INS + PIO, respectively. Some films required longer exposure intervals for detection of low level transcripts.

and GLUT4 (31). Multiple mechanisms exist by which hormones and other factors control the rate of cellular glucose uptake via these transporters. These include the rapid translocation of preexisting transporters from an intracellular pool to the plasma membrane, modulation of the intrinsic activity of preexisting plasma membrane glucose transporters, and stimulation of the synthesis of new transporters (32). We clearly observed increased transporter synthesis, since our results demonstrated increases in the expression of both GLUT1 and GLUT4 transporters on mRNA and protein levels (5- to 10-fold maximal enhancement for each). These results are in agreement with previous studies in which GLUT1 and GLUT4 mRNA and protein levels were shown to increase during differentiation of 3T3 preadipose cells (27, 33). Our observed increase in glucose transporter activity appeared to be somewhat greater in proportion than the combined increases in the synthesis of the two transporter proteins. This observation indicated that another mechanism, such as increased intrinsic activity of transporters, may also contribute to the effect. Such regulation of intrinsic transporter activity has previously been reported (34, 35). Inhibition of 3T3-L1 adipocyte protein synthesis by anisomycin, for instance, appeared to stimulate glucose transport primarily by enhancing the intrinsic catalytic activity of cell surface GLUT1, and to a lesser extent GLUT4 proteins (34). Further, treatment of 3T3-L1 preadipocytes with tumor necrosis factor-α reportedly increased glucose transport and GLUT1 transporter intrinsic activity (35). Alternatively, it is possible that the apparent difference in glucose transport activity and glucose transporter protein levels may be a consequence of our presentation of data as fold-enhancement relative to low level controls. As such, we may have some differences in detection sensitivity rather than absolute differences in the magnitude of changes.

Increased mRNA abundance can be attributed to enhanced RNA transcription and/or increased message stability. It was previously established that increases in the steady state level of several mRNAs during differentiation were accompanied by activation of specific gene transcription (36–39). This was particularly shown for mRNAs whose abundance was increased markedly (20- to 100-fold) during differentiation, including the aP2 and glycerolphosphate dehydrogenase genes (36). The same report showed no significant changes in the rates of transcription of mRNAs for which abundance was more moderately altered (2- to 4-fold) during differentiation, such as those encoding fructose-1,6-biphosphate, β-actin, and β-tubulin (36). In any case, most adipocyte mRNAs were far more abundant than would be predicted by their increased nuclear transcription rates. When increases in steady state mRNA levels cannot be attributed to changes in transcription, other levels of control such as mRNA stability likely contribute to the relative abundance of mRNAs during adipocyte differentiation. Since we observed moderate increases in GLUT1 and GLUT4 mRNA abundance (about 5-fold) with adipocyte differentiation, we compared mRNA half-lives in undifferentiated and differentiated cells using Act D (a transcription inhibitor) chase experiments. Differentiation of 3T3-F442A cells by insulin and/or pioglitazone dramatically increased the mRNA half-lives for GLUT1 and GLUT4 above their values in undifferentiated cells, *i.e.* from 1–2 h up to greater than 24 h. Such stabilization of these mRNAs with adipocyte differentiation correlated well with increases in the mRNA steady state levels. It is interesting to note that Actinomycin D itself has been reported in a few instances to have mRNA stabilizing effects (40–42). It would therefore be possible to extend our observations by conducting experiments using different transcription inhibitors such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole or thiolutin, or using a different method such as [^{3}H]uridine pulse to

verify message stability. However, our observed dramatic increase in the stability of transcripts encoding glucose transporters in adipocytes versus fibroblasts seems likely to remain, though there may be some differences in the absolute calculated half-lives.

Our findings represent the first study that directly associates increased steady state mRNA levels during adipocyte differentiation with increased mRNA stability. This process was, however, predicted earlier based on observed increases in mRNA transcript levels that could not be explained by increased gene transcription (36–38). Regulation of mRNA stability in such cells has, in fact, been reported for other conditions. Treatment of 3T3-F442A mature adipocytes with retinoic acid, for instance, specifically decreased the adipsin mRNA level (43). For such studies, the rate of adipsin gene transcription remained unchanged, whereas the half-life of adipsin mRNA was greatly shortened in retinoic acid-treated adipocytes as compared with untreated cells (37.6–7.3 h). Another example is the induction of GLUT1, as well as other immediate-early growth-related protooncogenes, in 3T3-L1 fibroblasts by treatment with tumor necrosis factor-α (26, 35) and 8-bromo-cAMP (44). Whereas transcriptional activation of immediate-early genes correlated well with subsequent accumulation of their respective mRNAs, increased GLUT1 mRNA was due to an apparent increase in the stability of this message (45 min to several hours) without changes in its transcription. Another study showed that increased GLUT1 mRNA abundance by chronic exposure of L6 myocytes to insulin was due to increased transcription as well as prolonged half-life (2–5 h) (45).

In general, the stabilization/destabilization of mRNAs in response to biological and pharmacological stimuli has been recognized as an important posttranscriptional step for regulation of gene expression (46–49). Despite that, the mechanisms underlying such processes, including the signals that trigger mRNA degradation or stabilization, the structural elements of the RNA that are recognized by degradative enzymes or stabilization factors, as well as the enzymes or other *trans*-acting factors themselves, are largely unknown (46, 49–52). Interestingly, a recent report indicated that the 3′-untranslated region of GLUT1 mRNA contains a single copy of the destabilizing AUUUA motif in the context of an AU-rich region (26). The stability of GLUT1 mRNA was found to be partially controlled by its interaction with a sequence-specific mRNA binding protein, the adenosine-uridine binding factor which was speculated to mediate mRNA stabilization by blocking the AU-destabilizing motifs (26). We therefore propose that increasing mRNA abundance during differentiation by increasing message stability presents an interesting phenomenon awaiting further examination. Future efforts should be particularly directed toward identifying common mRNA sequences that may function as stabilizing elements in the differentiation-induced mRNAs as well as identifying their regulatory binding proteins.

Acknowledgments

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L-Glutamic Acid γ-Monohydroxamate

A POTENTIATOR OF VANADIUM-EVOKED GLUCOSE METABOLISM *IN VITRO* AND *IN VIVO**

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We report that the vanadium ligand L-Glu(γ)HXM potentiates the capacity of free vanadium ions to activate glucose uptake and glucose metabolism in rat adipocytes *in vitro* (by 4–5-fold) and to lower blood glucose levels in hyperglycemic rats *in vivo* (by 5–7-fold). A molar ratio of two L-Glu(γ)HXM molecules to one vanadium ion was most effective. Unlike other vanadium ligands that potentiate the insulinomimetic actions of vanadium, L-Glu(γ)HXM partially activated lipogenesis in rat adipocytes in the absence of exogenous vanadium. This effect was not manifested by D-Glu(γ)HXM. At 10–20 μM L-Glu(γ)HXM, lipogenesis was activated 9–21%. This effect was approximately 9-fold higher (140 ± 15% of maximal insulin response) in adipocytes derived from rats that had been treated with vanadium for several days. Titration of vanadium(IV) with L-Glu(γ)HXM led to a rapid decrease in the absorbance of vanadium(IV) at 765 nm, and ^{51}V NMR spectroscopy revealed that the chemical shift of vanadium(IV) at −490 ppm disappeared with the appearance of a signal characteristic to vanadium(V) (−530 ppm) upon adding one equivalent of L-Glu(γ)HXM. In summary, L-Glu(γ)HXM is highly active in potentiating vanadium-activated glucose metabolism *in vitro* and *in vivo* and facilitating glucose metabolism in rat adipocytes in the absence of exogenous vanadium probably through conversion of trace intracellular vanadium into an active insulinomimetic compound. We propose that the active species is either a 1:1 or 2:1 L-Glu(γ)HXM vanadium complex in which the endogenous vanadium(IV) has been altered to vanadium(V). Finally we demonstrate that L-Glu(γ)HXM- and L-Glu(γ)HXM·vanadium-evoked lipogenesis is arrested by wortmannin and that activation of glucose uptake in rat adipocytes is because of enhanced translocation of GLUT4 from low density microsomes to the plasma membrane.

Intensive studies have been carried out during the last two decades on the insulinomimetic effects of vanadium (1–4). Vanadium salts mimic most of the effects of insulin on the main target tissues of the hormone *in vitro* and also induce normoglycemia and improve glucose homeostasis in insulin-deficient (5–7) and insulin-resistant diabetic rodents *in vivo* (5–8). On the basic research frontier, data continue to accumulate showing that vanadium salts manifest their insulin-like metabolic effects through alternative pathways not involving insulin receptor tyrosine kinase activation or phosphorylation of insulin receptor substrate 1 (9–19). The key events of this backup system appear to involve inhibition of protein-phosphotyrosine phosphatases and activation of nonreceptor protein-tyrosine kinases (20–23).

Vanadium salts are seriously considered as a possible treatment for diabetes, and several clinical studies have already been performed. In those studies, because of its toxicity, only low doses of vanadium (2 mg/kg/day) were used. Although ~20-fold lower than doses used in most animal studies, several beneficial effects were observed and documented (24–26). Any manipulation to elevate the insulinomimetic efficacy of vanadium without increasing its toxicity is of major clinical interest for the future care of diabetes (reviewed in Ref. 27).

Organically chelated vanadium compounds, such as vanadium·acetylacetonate and vanadium·RL-252,[1] are more potent than free vanadium in facilitating insulin-like effects in rat adipocytes (28, 29). Similarly, chelated vanadium compounds such as bis(maltolato)oxovanadium and bis(picolinato)oxovanadium are more effective than free vanadium in reducing circulating glucose levels in hyperglycemic streptozocin-treated rats (30–33).

In the wake of these findings, we have continued our search for more effective vanadium binding agents. Of special interest to us were vanadium chelators that synergize with vanadium both *in vivo* (*i.e.* in streptozocin rats) and *in vitro* (*i.e.* in isolated rat adipocytes) and therefore enable us to gain insight into the basic mechanism(s) by which such compounds potentiate the insulinomimetic activity of vanadium. Specifically, we have studied hydroxamic acid derivatives. These compounds are involved in the microbial transport of iron and are therefore applied therapeutically in conditions of iron deficiency (34). They are also inhibitors of urease activity and have been used in the treatment of hepatic coma. Monoamino acid hydroxamates are simple, nontoxic derivatives of amino acids. D-Aspartic acid β-hydroxamate was shown to have antitumoral activity on murine leukemia L5178Y, both *in vitro* and *in vivo*, and is

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¶ Partial fulfillment of the requirements for the Ph.D. degree.
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[1] The abbreviations used are: RL-252, $[(CH_2)_2\text{-}C\text{-}[CH_2O\text{-}(CH_2)_2\text{-}CONHCH(iBu)CONOHCH_2]_2]$; L-Glu(γ)HXM, L-glutamic acid γ-monohydroxamate; GLUT4, glucose transporter 4; PM, plasma membranes; LDM, low density microsomes; BSA, bovine serum albumin; $VOCl_2$, vanadyl dichloride; $NaVO_3$, sodium metavanadate.

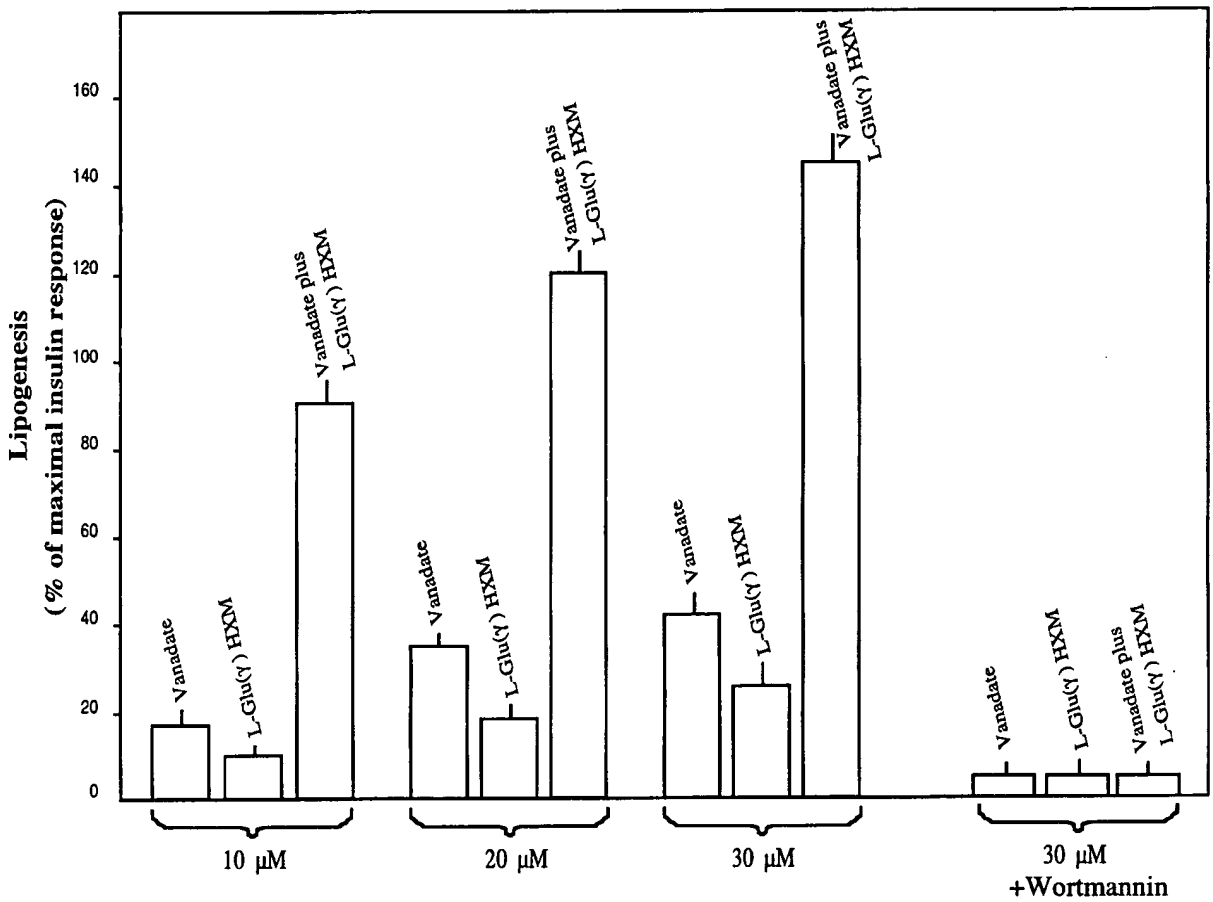

FIG. 1. **Increase in the lipogenic capacity of vanadium(V) following the addition of L-glutamic acid(γ)monohydroxamate.** Freshly prepared rat adipocytes (3×10^5 cells/ml) suspended in KRB buffer, pH 7.4, containing 0.7% BSA were preincubated for 10 min with the indicated concentrations of $NaVO_3$, free Glu(γ)HXM, and a 1:1 complex of L-Glu(γ)HXM·$NaVO_3$. The cells were then supplemented with [U-^{14}C]glucose, and lipogenesis was performed for 2 h at 37 °C. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

active against Friend leukemia cells *in vitro* as well (35). L-Glu(γ)HXM is cytotoxic against L1210 cells in culture and remarkably antitumoral against L1210 leukemia and B16 melanoma *in vivo* (35, 36).

EXPERIMENTAL PROCEDURES

Materials—D-[U-^{14}C]glucose and 2-deoxy-D-[G-^{3}H]glucose were purchased from NEN Life Science Products. Collagenase type I (134 units/mg) was obtained from Worthington. Porcine insulin was purchased from Eli Lilly Co. (Indianapolis, IN). Phloretin, 2-deoxyglucose, L-glutamic acid(γ)-monohydroxamate, L-aspartic acid(β)monohydroxamate, glycine hydroxamate, L-isoleucine(α)hydroxamate, and L-tyrosine(α)hydroxamate were purchased from Sigma. RL-252 was prepared and characterized as described earlier (28).

Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) contained 110 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$. Krebs-Ringer bicarbonate HEPES (KRBH) buffer (pH 7.4) consisted of 117 mM NaCl, 10 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM KH_2PO_4, 30 mM HEPES. All other chemicals and reagents used in this study were of analytical grade.

Streptozocin-treated Rats—Diabetes was induced by a single intravenous injection of a freshly prepared solution of streptozocin (55 mg/kg body weight) in 0.1 M citrate buffer, pH 4.5 (9). The effect of the L-Glu(γ)HXM·vanadium complex on blood glucose level was determined 8 days after induction of diabetes by streptozocin.

Cell Preparation and Bioassays—Rat adipocytes were prepared from the fat pads of male Wistar rats (130–150 g) by collagenase digestion according to the method of Rodbell (37). Cell preparations showed more than 95% viability by Trypan blue exclusion at least 3 h after digestion. All bioassays were performed as described in figure legends. Glucose transport was carried out using 2-deoxy-D-[G-^{3}H]glucose uptake (38), and lipogenesis (the incorporation of U-^{14}C-labeled glucose into lipids) was performed according to Moody *et al.* (39). Briefly, freshly prepared rat adipocytes were suspended in KRBH, 0.7% BSA buffer and divided into about 50 plastic vials. Each vial contained 0.5 ml of adipocyte suspension (about 1.5×10^5 cells). These were incubated for 2 h at 37 °C under an atmosphere of 95% O_2, 5% CO_2 with 0.16 mM [U-^{14}C]glucose. Each assay contained vials with and without 17 nM insulin and the various test compounds. Lipogenesis was terminated by adding toluene-based scintillation fluid, and the extracted lipids were counted (39). Results are expressed as a percent of maximal insulin response. Only assays in which insulin activated lipogenesis 5–6-fold above basal (basal ~4000 cpm/1.5×10^5 cells/2 h, $V_{insulin}$ = 20,000–24,000 cpm/1.5×10^5 cells/2 h) were taken into consideration. Insulin activated lipogenesis in this assay at an ED_{50} value of 33 ± 3 pM. A concentration of 0.3 nM insulin and above already facilitated maximal (100%) response (*i.e.* Ref. 16). All assays were performed in duplicate or triplicate.

Western Immunoblot Analysis of GLUT4 in Subcellular Membranes Following Stimulation of Rat Adipocytes—Adipocytes prepared from 6-week-old rats were incubated with and without insulin and with L-Glu(γ)HXM alone and complexed with vanadate as specified in the figure. Cells were then homogenized and fractionated to low density microsomal membrane (LDM) and plasma membrane (PM) fractions by differential ultracentrifugation according to Ref. 40. Membrane proteins were then solubilized in sample buffer for 30 min at 25 °C, resolved on 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper, and immunoblotted with anti-GLUT4 antisera (41). Visualization was performed by phosphoimaging. The relative intensity of bands corresponding to GLUT4 was quantitated using MacBas 1000.

^{51}V NMR Spectroscopy—The ^{51}V NMR spectra were recorded on a 200-MHz Bruker WPS4 (4.7T) spectrometer. Spectrum width of 16,000

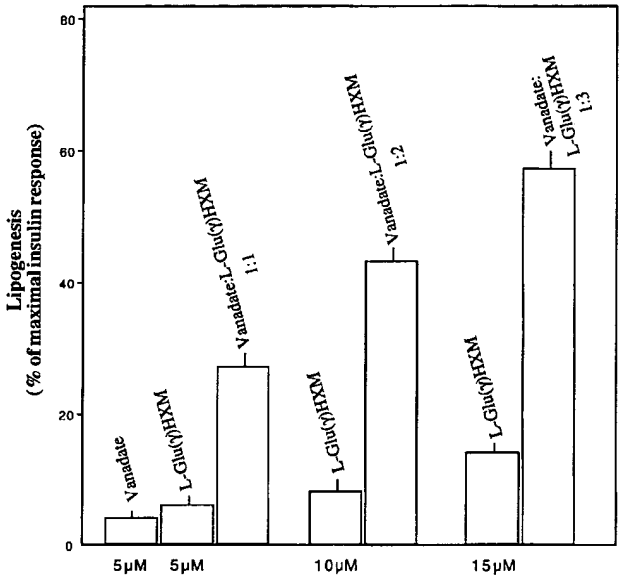

FIG. 2. **Stimulation of lipogenesis at varying molar ratios of L-Glu(γ)HXM to vanadium(V).** Freshly prepared rat adipocytes (3×10^5 cells/ml) suspended in KRB buffer, pH 7.4, containing 0.7% BSA were preincubated for 10 min with the indicated concentrations of 1:1 to 3:1 molar stoichiometry of L-Glu(γ)HXM to $NaVO_3$ or with free $NaVO_3$(V) and free L-Glu(γ)HXM. The cells were then supplemented with [U-^{14}C]glucose, and lipogenesis was performed for 2 h at 37 °C. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

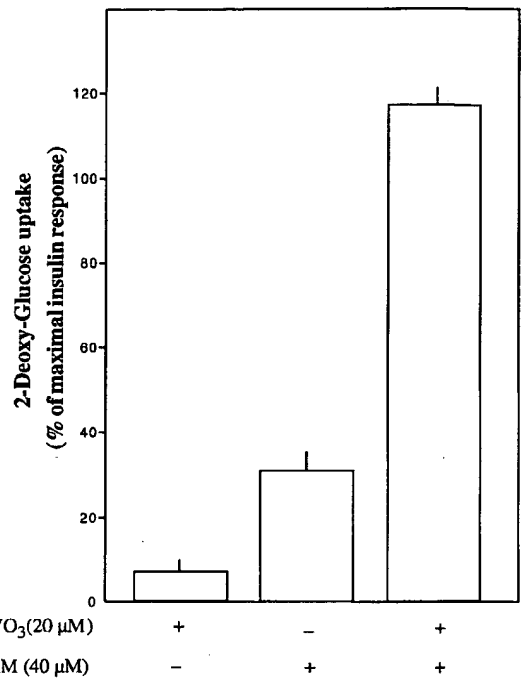

FIG. 3. **Potentiation of hexose uptake by L-Glu(γ)HXM·vanadium (2:1).** Adipocytes (2×10^6 cells/ml) suspended in KRBH buffer containing 1% BSA were preincubated in the presence and absence of insulin (17 nM), sodium metavanadate (20 μM), L-Glu(γ)HXM (40 μM), and their combination (at 1:2 molar stoichiometry). Aliquots (70 μl) were transferred into tubes containing 2-deoxy-D-[6-^{3}H]glucose (0.1 mM final concentration). Phloretin (0.1 mM) was added after 3 min for transport termination. This was followed by centrifugation of aliquots through a silicone layer.

H_3, a 90° pulse angle, and an accumulation time of 0.28 were used. The chemical shifts are reported relative to the external reference standard $VOCl_2$ (−490 ppm).

RESULTS

L-Glutamic Acid(γ)Monohydroxamate Potentiates Vanadium-evoked Lipogenesis in Rat Adipocytes—In this set of experiments, rat adipocytes were incubated for 10–20 min with submaximal concentrations of vanadate (10–30 μM), L-Glu(γ)HXM (10–30 μM), or an equimolar combination of them. The capacity to activate lipogenesis relative to insulin was then determined. As shown in Fig. 1, the combination was highly synergistic. For example, at 10 μM vanadate or L-Glu(γ)HXM, lipogenesis was 17 ± 3 and 9 ± 2%, respectively, whereas the combination produced a marked incredible 93 ± 4% activation of maximal insulin response. At 20 μM, the extent of lipogenesis was 37 ± 3, 20 ± 3, and 121 ± 7%, and at 30 μM, it was 42 ± 4, 23 ± 4, and 143 ± 7% of maximal. Wortmannin (100 nM), an inhibitor of phosphatidylinositol 3-kinase, fully blocked the activating effects of vanadate, L-Glu(γ)HXM, and its combination with vanadate (Fig. 1, *right columns*). Thus L-Glu(γ)HXM potentiated vanadate-evoked lipogenesis about 3.5–5-fold; the higher concentrations reached a level that is about 140% of that achieved by saturating concentrations of insulin or vanadate. A finding of significant interest to us was the ability of L-Glu(γ)HXM to partially activate lipogenesis even in the absence of exogenous vanadium (Fig. 1). This finding is examined in great detail in connection with Fig. 6.

In Fig. 2, lipogenesis in rat adipocytes was evaluated at a fixed, low concentration of vanadate (5 μM) with increasing concentrations of L-Glu(γ)HXM. Lipogenesis was negligible at 5 μM vanadate or L-Glu(γ)HXM alone (4–6% of maximal insulin effect) but is augmented to 27.0 ± 3% when they were given in combination (at a molar stoichiometry of 1:1). At 2:1 and 3:1 Glu(γ)HXM·vanadium molar stoichiometry, lipogenesis expanded to 43 and 57%, respectively, of maximal response. Thus a substantial synergistic effect is obtained at a 1:1 molar ratio and is increased further at a 2:1 molar stoichiometry and even higher, though much less pronounced, at a 3:1 molar ratio (Fig. 2).

L-Glu(γ)HXM Potentiates Vanadate-evoked Glucose Uptake—Fig. 3 shows activation of 2-deoxyglucose uptake by low concentrations of vanadate (20 μM), L-Glu(γ)HXM (40 μM), and by the 2:1 molar combination, of them. 2-Deoxyglucose undergoes insulin- or vanadate-evoked influx into the cell via the same transporters as glucose and is phosphorylated *in situ* to 2-deoxyglucose 6-phosphate with no further metabolism (42, 43). Therefore, this measurement reflects an effect on glucose entry into the cell in a manner largely independent of the metabolism of the endogenous saccharide. Vanadate (20 μM) and L-Glu(γ)HXM (40 μM) affected 2-deoxyglucose uptake of 7 ± 0.7 and 31 ± 4% of maximal insulin effect, respectively. Together they caused 2-deoxyglucose uptake 117 ± 9% of maximal insulin response (Fig. 3).

L-Glu(γ)HXM Alone and L-Glu(γ)HXM·Vanadate Lead to Translocation of GLUT4 from LDM to PM Fractions in Rat Adipocytes—Incubation of rat adipocytes with L-Glu(γ)HXM and L-Glu(γ)HXM·vanadate led to a decrease in the content of GLUT4 in the LDM fraction and an increase in the PM fraction (Fig. 4). The decrease in GLUT4 content in the low density lipoprotein fraction amounted to 32 ± 3, 3 ± 1, and 68 ± 5% of maximal insulin response upon incubating the cells with L-Glu(γ)HXM (40 μM), vanadate (20 μM, not shown), and the combination, respectively (calculated from Fig. 4). Under similar experimental conditions, L-Glu(γ)HXM, vanadate, and the combination activated 2-deoxyglucose uptake to an extent of 31 ± 4, 7 ± 0.7, and 117 ± 9% of maximal insulin response (Fig. 3), suggesting a contributing effect of the complex to glucose influx in addition to its effect in recruiting GLUT4 transporters

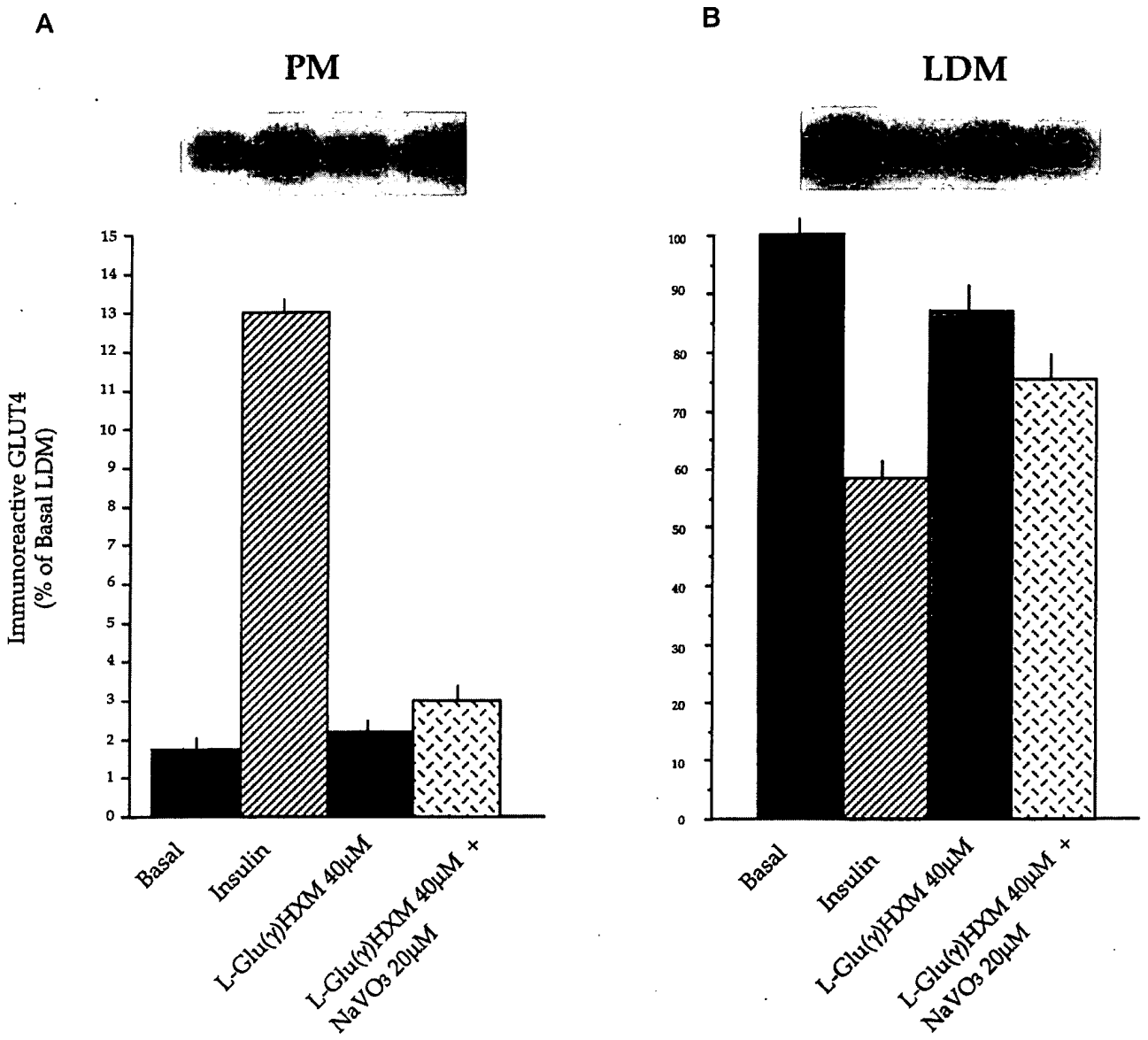

FIG. 4. **L-Glu(γ)HXM alone or complexed with vanadate induces translocation of GLUT4 from LDM to PM fraction in rat adipocytes.** Rat adipocytes were incubated for 30 min at 37 °C in the presence and the absence of insulin (17 nM) and the indicated concentrations of L-Glu(γ)HXM or L-Glu(γ)HXM-vanadate. Cells were then homogenized and fractionated to PM and LDM by differential ultracentrifugation, and GLUT4 protein was identified by Western immunoblot analysis ("Experimental Procedures"). Immunoreactive GLUT4 proteins were visualized by phosphoimaging (*top panels*) and were quantitated using MacBas 1000 software (histograms, *bottom panels*).

from the low density lipoprotein to the PM fraction.[2]

L-Glu(γ)HXM-Vanadate Normalizes Blood Glucose Levels in Streptozocin-treated Diabetic Rats—In the experiments summarized in Fig. 5, streptozocin-treated rats received intraperitoneally sodium metavanadate (0.05 mmol/kg body weight), L-Glu(γ)HXM (0.1 mmol/kg body weight), or a combination of the two compounds 8 days after the induction of diabetes. As shown in the figure, vanadate and L-Glu(γ)HXM, at these concentrations, had a rather minor effect in reducing the high circulating glucose levels characterizing these hyperglycemic rats. The combination, however, was highly efficient at normalizing blood glucose levels. Normoglycemia was evident 1 day after the first administration and remained so following two more administrations. The glucose levels then remained close to normal for the next 3 days (Fig. 5).

Activation of Lipogenesis in Rat Adipocytes by L-Glu(γ)HXM in the Absence of Exogeneous Vanadium—L-glutamic acid(γ)HXM also activated lipogenesis in the absence of added vanadium, and this effect was studied in detail (Fig. 6). The dose-response curve (Fig. 6*A*) indicates that activation is already evident at 5 μM L-Glu(γ)HXM and that higher concentrations reach a level of 40 ± 7% of maximal insulin response (median effective dose = 35 ± 4 μM). Other amino acid hydroxamates such as L-Tyr(α)HXM, Gly(α)HXM, and L-Ile(α)HXM also activated lipogenesis, but they were considerably less potent (ED_{50} = 250 ± 30 μM, 40 ± 5% of maximal insulin effect). L-Aspartic acid β-monohydroxamate showed higher lipogenic activity compared with the α-amino acid hydroxamates and was slightly less potent than L-Glu(γ)HXM (ED_{50} = 45 ± 7 μM, Fig. 6*B*). *N*-acetyl-L-Glu(γ)HXM and L-Glu(γ)HXM-α-methyl ester were virtually ineffective, indicating the need for a free α-amino and, to a somewhat lesser extent, a free

[2] I. Goldwaser, J. Li, E. Gershonov, M. Armoni, E. Karnieli, M. Fridkin, and Y. Shechter, manuscript in preparation.

α-carboxyl moiety for the activation of lipogenesis by L-Glu(γ)HXM in the rat adipose cell (Fig. 6C). Stereospecificity appears crucial as well, because the D-isomer of Glu(γ)HXM was ineffective. All these findings indicate that activation of lipogenesis by L-Glu(γ)HXM depends on a specific entry of this L-amino acid analog into the adipose cell. Further investigation has led us to suggest that L-Glu(γ)HXM enters the adipose cell primarily through the non-Na^+-dependent glutamine transport system.[2]

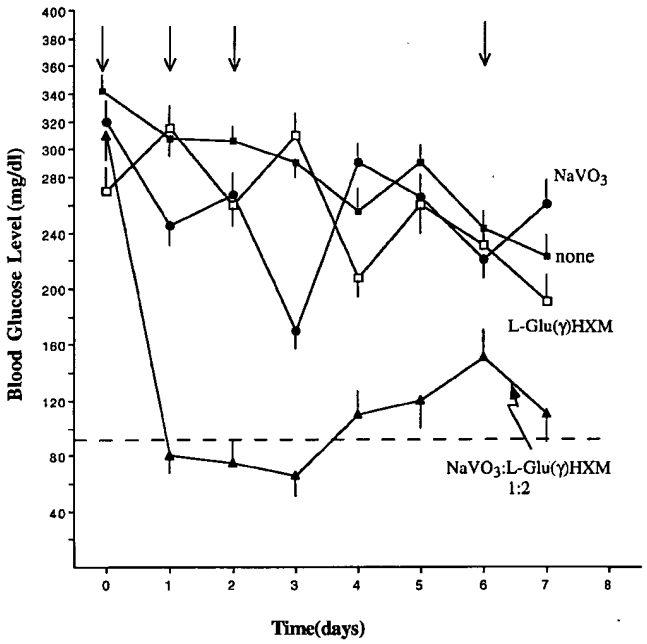

FIG. 5. **Effect of L-Glu(γ)HXM-vanadate administration on blood glucose levels of streptozocin-treated rats.** Male Wistar rats, 8 days after induction of diabetes (circulating glucose levels 310–340 mg/dalton), were divided into several groups. At the time points indicated by the *arrows* (intraperitoneal, at 11:00 a.m.), groups of diabetic rats received either vanadate (0.05 mmol/kg body weight ●), L-Glu(γ)HXM (0.1 mmol/kg body weight □), L-Glu(γ)HXM (0.1 mmol/kg) and vanadate (0.05 mmol/kg, ▲), or none (■). Circulating glucose levels were determined daily (at 8.00 a.m.). Each point in the figure represents the arithmetic mean of plasma glucose for 5 rats. The *dashed line* indicates the arithmetic mean of plasma glucose of control healthy male Wistar rats.

Several organic chelators, which potentiate the insulinomimetic activity of vanadium either *in vitro* or *in vivo*, have been documented. These include acetylacetonate (29), maltol (30, 31), picolinate (32, 33), and RL-252 (28). In Fig. 6D, we have examined whether they are capable of activating lipogenesis in the absence of exogenous vanadium. Unlike L-Glu(γ)HXM, none of these agents were able to activate lipogenesis in the rat adipose cell at concentrations of 100 μM (Fig. 6D) or lower (not shown).

Extensive Potentiation of L-Glu(γ)HXM-evoked Lipogenesis in Rat Adipocytes in Vitro Following Enrichment with Vanadium in Vivo—The findings presented in Figs. 1–4 have taught us that L-Glu(γ)HXM potentiates the insulinomimetic potency of vanadium and that activation of lipogenesis by L-Glu(γ)HXM alone never exceeds 40 ± 7% of maximal insulin effect (Fig. 6). To examine whether L-Glu(γ)HXM-evoked lipogenesis can be affected by the level of intracellular vanadium, a group of male Wistar rats received daily subcutaneous administrations of vanadate (0.1 mmol/kg/day) over a period of 5 days to raise the level of endogenous vanadium. Rats were then sacrificed 7 h after the last administration. Adipocytes were prepared, and the effect of L-Glu(γ)HXM on lipogenesis was compared with that in nontreated freshly prepared adipocytes. As shown in Fig. 7, vanadium-enriched adipocytes became dramatically sensitive to L-Glu(γ)HXM-evoked lipogenesis. This was valid both in terms of a leftward shift in the dose-response curve to L-Glu(γ)HXM (ED_{50} = 6.4 ± 0.3 μM *versus* ED_{50} = 35 ± 4 μM in control adipocytes) and in terms of the degree of lipogenesis (145 ± 15 *versus* 40 ± 7% of maximal insulin response, *i.e.* Fig. 6). At 10 μM, L-Glu(γ)HXM already stimulated lipogenesis and amounted to 120% of maximal insulin effect in the vanadium-enriched adipose cells (as opposed to only 8.0 ± 1.5% in control adipocytes) (Fig. 7).

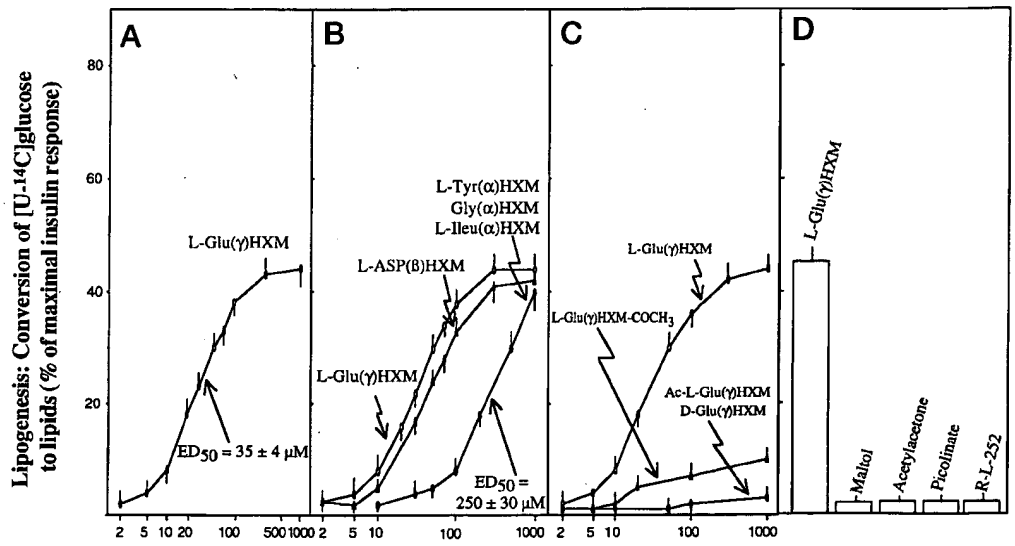

FIG. 6. **Activation of lipogenesis by L-Glu(γ)HXM in the absence of exogenous vanadium.** Comparison to other amino acid(α)hydroxamates and ineffectiveness of the D-isomer and of chemically modified L-Glu(γ)HXM derivatives. Freshly prepared adipocytes (3×10^5 cells/ml) suspended in KRB buffer, pH 7.4, containing 0.7% BSA were preincubated for 10 min with the indicated concentrations of the various test compounds. The cells were then supplemented with [U-^{14}C]glucose (final concentration 0.16 mM), and lipogenesis was performed for 2 h at 37 °C. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

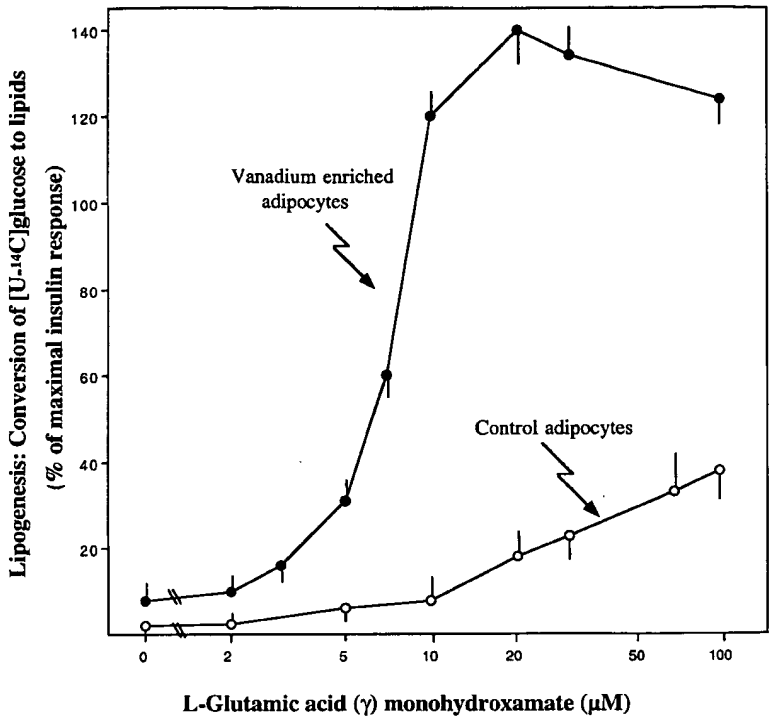

FIG. 7. **Activation of lipogenesis by L-Glu(γ)HXM.** Comparison between normal adipocytes and vanadium-enriched adipocytes. Male Wistar rats received daily subcutaneously injected $NaVO_3$ (0.1mmol/kg/day) for 5 days (called enriched vanadium rats). The rats were then sacrificed (7 h after the last administration). Lipogenesis was performed comparing the freshly prepared rat adipocytes (3×10^5 cells/ml) from nonenriched vanadium rats with the enriched ones suspended in KRB buffer, pH 7.4, containing 0.7% BSA. The cells were preincubated for 10 min with the indicated concentrations of L-Glu(γ)HXM. The cells were then supplemented with [U-^{14}C]glucose, and lipogenesis was performed for 2 h at 37 °C. Radioactivity incorporated into extracted lipids was then determined. Maximal response (100%) is that obtained in the presence of 17 nM insulin.

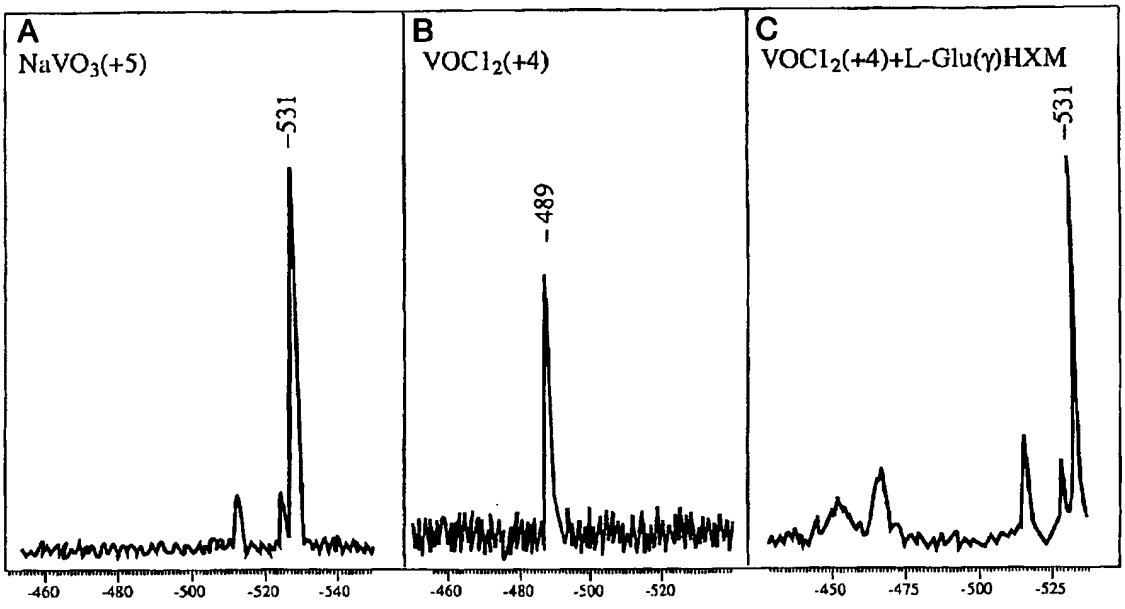

FIG. 8. **^{51}V NMR spectra of vanadium(V), vanadium(IV), and a mixture of vanadium(IV) with L-Glu(γ)monohydroxamate.** *A*, ^{51}V NMR spectrum of sodium metavanadate (20 mM, pH 7.1); *B*, ^{51}V NMR spectrum of vanadium dichloride(IV) (20 mM, pH 6.8); *C*, ^{51}V NMR spectrum of a mixture (1:1 molar ratio) of $VOCl_2$(IV) and L-Glu(γ)HXM (20 mM, pH 7.0). Spectra were monitored with fresh solutions. In *C*, the spectrum was monitored 5 min after the addition of L-Glu(γ)HXM to $VOCl_2$.

Spectroscopic Studies—Previously we found in cell-free experiments that vanadium(IV), at neutral pH values, undergoes slow spontaneous oxidation to vanadium(V). This occurs similarly in the presence of 10 mM reduced glutathione, an ineffectual reductant of vanadium(V), at neutral pH values with a $t_{1/2}$ value of 1 ± 0.1 h at 25 °C (29). The results summarized in Fig. 8 show the V^{51} NMR spectra of vanadium dichloride(IV) at pH 7.0 prior to and after the addition of L-Glu(γ)HXM. Vanadium dichloride(IV) appeared as a single peak with a chemical shift of −490 ppm in its ^{51}V spectrum, indicating one main species present at >95% purity. Upon the addition of L-Glu(γ)HXM (1 equivalent), the chemical shift of vanadium(IV) at −490 ppm disappeared within minutes and the principal chemical shift characterizing vanadium(V) at −530 ppm appeared (Fig. 8).

Vanadium(IV) (*i.e.* vanadyl sulphate or $VOCl_2$) has a characteristic "blue" absorbance with $\epsilon_{765\ nm} = 14 \pm 0.3$, whereas vanadium(V) does not absorb at all at this wavelength (29). The addition of 2–3 equivalents of L-Glu(γ)HXM to $VOCl_2$(IV) (50 mM at pH 7.5) led rapidly to a near total decrease in vanadium(IV) absorbance at 765 nm (Fig. 9). Fig. 8*B* depicts complex formation as a function of the pH in the range of pH 2–9. Decrease is minimal at pH 4.0, quite significant at pH 5.0,

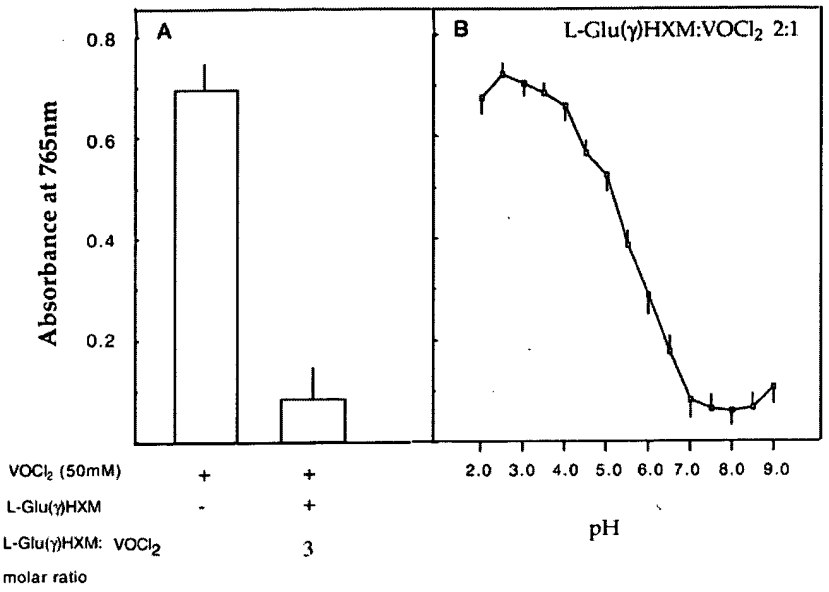

FIG. 9. **Decrease in absorbance of vanadium(IV) at 765 nm upon addition of L-Glu(γ)HXM.** Effect of pH: *A, left column,* absorbance of $VOCl_2$ alone (50 mM in H_2O); *right column,* absorbance of $VOCl_2$ (50 mM) and L-Glu(γ)HXM (150 mM) titrated with $NaHCO_3$ to pH 7.4. *B,* samples of $VOCl_2$ (50 mM) and L-Glu(γ)HXM (100 mM) in H_2O were titrated either with HCl or with $NaHCO_3$ before absorbance at 765 nm and were monitored to obtain the pH values indicated in the figure. L-Glu(γ)HXM alone does not absorb at 765 nm. Vanadium dichloride alone, which tends to precipitate at neutral pH values, remains completely soluble at all pH values in the presence of two or more equivalents of L-Glu(γ)HXM.

half-maximal at pH 5.7, and reaches a stable plateau at pH range 7–9 (Fig. 9*B*).

DISCUSSION

It has been consistently observed that chelated vanadium compounds are more potent than the free metaloxide in facilitating the metabolic actions of insulin. This was demonstrated *in vitro* with systems like rat adipocytes, as well as in diabetic rodents such as streptozocin-treated hyperglycemic rats (28–33, 44). Because of the variations in the experimental models used, the oxidation state of vanadium applied, and the different administration modes, the basis for the higher insulinomimetic potencies of complexed vanadium remained rather speculative. Because this topic has immediate therapeutic relevance, we looked for new vanadium chelators characterized by: (*a*) higher synergistic potencies than previously documented for vanadium chelators with respect to vanadium-evoked glucose uptake and glucose metabolism both *in vitro* and in diabetic rats *in vivo*, (*b*) low indices of toxicity, and (*c*) reasonable solubility in aqueous, neutral media after complexation with vanadium.

In this study, we have introduced the L-isomer of glutamic acid(γ)monohydroxamate as it satisfactorily fulfilled the above criteria. It potentiated vanadium-activated hexose uptake, glucose metabolism, and recruitment of GLUT4 transporters from LDM to PM fractions (Figs. 1–4). *In vivo* it potentiated the efficacy of vanadium to lower blood glucose levels in streptozocin rats (Fig. 5). This amino acid analog has negligible toxicity in mammals.[2] Both L-Glu(γ)HXM alone and its complexes with vanadium are fairly soluble in aqueous media at neutral pH values. An important finding was that L-Glu(γ)HXM alone, in the absence of exogenous vanadium, showed a reasonable amount of insulinomimetic activity in that it activated glucose uptake and glucose metabolism in the rat adipose cell (Figs. 1–3). Further investigation revealed that this activating effect is unique to the L-isomer of Glu(γ)HXM but is not facilitated by the D-isomer. Nonmodified α-amino and α-carboxyl moieties appear essential. This intrinsic activity is exclusive to L-Glu(γ)HXM not being shared by any of the other vanadium chelators that potentiate the actions of vanadium *in vivo* or *in vitro* (Fig. 6, *A–D*, and Refs. 28–33). Our assumption that L-Glu(γ)HXM permeates into the cell interior and transforms the "dormant" intracellular vanadium pool into an insulinomimetic-activated species gains credence from the dramatic sensitization of vanadium-enriched adipocytes to L-Glu(γ)HXM-evoked lipogenesis (Fig. 7).

It should be mentioned at this point that because of the extreme complexity of aqueous vanadium chemistry (reviewed in Refs. 46–49), the intracellular milieu of the mammalian cell is still "a black box" with respect to the state and the form of entered vanadium. With the endogenously present vanadium pool, experiments have shown that it exists mostly as vanadium(IV), though some researchers may wonder even about this experimental finding because vanadium in its IV oxidation state is only stable at acidic pH values (pH < 3.0) and readily oxidizes to vanadium(V) at neutral pH even in the presence of high glutathione concentrations (28, 46). The intracellular vanadium pool, however, can be preserved in its IV oxidation form at neutral pH values if it is chelated by ascorbic acid (not shown) or to endogenous proteins (50, 51). At the low physiological level of intracellular vanadium, the cell should have the capacity to chelate all the endogenous vanadium.

Our experimental findings that L-Glu(γ)HXM alone enhances glucose uptake and glucose metabolism (Figs. 1 and 2) together with the apparent rapid conversion of vanadium(IV) to vanadium(V) upon complexation (Figs. 8 and 9) strongly support the contention that vanadium(V) rather than vanadium(IV), and in a chelated form, is the active insulinomimetic species that facilitates the activation of glucose uptake and its metabolism in rat adipocytes. Although most of our previous cell-free experiments support this conclusion, we were not fully convinced prior to the completion of this study. This is because protein phosphotyrosine phosphatases (with *p*-nitrophenylphosphate as a substrate) are inhibited by both vanadium(IV) and vanadium(V), free or chelated, at nearly the same concentrations (see Ref. 52). On the other hand, adipose nonreceptor protein-tyrosine kinases, whether cytosolic or membranal, are with one exception activated by vanadium(V) but not at all by vanadium(IV) (22, 23). We have only observed vanadium(IV)-evoked activation of nonreceptor protein-tyrosine kinases when membranal protein phosphotyrosine phosphatases were extracted with Triton X-100 and added to the cytosolic protein-tyrosine kinase fraction (29). These experimental conditions, however, are not likely to occur in the intact cell system. For example, broken plasma membrane fragments (or deoxycholate-treated membranal fragments) did not sup-

port activation of cytosolic protein-tyrosine kinases in the presence of vanadium(IV) (29).

In summary, L-Glu(γ)HXM appears superior to previously documented organic chelators of vanadium in potentiating its activation of glucose uptake and glucose metabolism *in vitro* and *in vivo*. Taken together with earlier studies, this may be attributed to one or more of the following: (*a*) increased efficiency of this specific combination to permeate into cells or tissues; (*b*) a favorable 5-coordinated, rather than octahedral topography of this complex in an aqueous, neutral environment (Ref. 50);[2] and/or (*c*) higher intracellular stability of the L-Glu(γ)HXM-vanadium complex. Finally, we have recently observed that vanadate does not inhibit alkaline phosphatase in the presence of L-Glu(γ)HXM.[2] This inhibitory effect of vanadate (53) is undesirable from our point of view as it may contribute to vanadium toxicity in mammals, but not to the efficacy of vanadium to manifest the metabolic actions of insulin (reviewed in Ref. 54). This and other basic and diabetological aspects raised here are being further investigated.

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Evidence That Glucose Metabolism Regulates Leptin Secretion from Cultured Rat Adipocytes*

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ABSTRACT

Circulating leptin secreted from adipocytes is correlated with fat mass and plasma insulin concentrations in humans and rodents. Plasma leptin, insulin, and glucose decrease during fasting and increase after refeeding; however, the underlying mechanisms regulating the changes of leptin secretion are not known. To investigate the role of insulin-stimulated glucose metabolism in the regulation of leptin secretion, we examined the effects of insulin and inhibitors of glucose transport and metabolism on leptin secretion from rat adipocytes in primary culture. Insulin (0.16–16 nM) increased leptin secretion over 96 h; however, the increase in leptin was more closely related to the amount of glucose taken up by the adipocytes ($r = 0.64$; $P < 0.0001$) than to the insulin concentration *per se* ($r = 0.20$; $P < 0.28$), suggesting a role for glucose transport and/or metabolism in regulating leptin secretion.

2-Deoxy-D-glucose (2-DG), a competitive inhibitor of glucose transport and phosphorylation, caused a concentration-dependent (2–50 mg/dl) inhibition of leptin release in the presence of 1.6 nM insulin. The inhibitory effect of 2-DG was reversed by high concentrations of glucose. Two other inhibitors of glucose transport, phloretin (0.05–0.25 mM) and cytochalasin-B (0.5–50 μM), also inhibited leptin secretion. Inhibition of leptin secretion by these agents was proportional to the inhibition of glucose uptake ($r = 0.60$ to 0.86; all $P < 0.01$). Two inhibitors of glycolysis, iodoacetate (0.005–1.0 mM) and sodium fluoride (0.1–5 mM), produced concentration-dependent inhibition of leptin secretion in the presence of 1.6 nM insulin. In addition, both 2-DG and sodium fluoride markedly decreased the leptin (*ob*) messenger RNA content of cultured adipocytes, but did not affect 18S ribosomal RNA content.

We conclude that glucose transport and metabolism are important factors in the regulation of leptin expression and secretion and that the effect of insulin to increase adipocyte glucose utilization is likely to contribute to insulin-stimulated leptin secretion. Thus, *in vivo*, decreased adipose glucose metabolism may be one mechanism by which fasting decreases circulating leptin, whereas increased adipose glucose metabolism would increase leptin after refeeding. (*Endocrinology* **139:** 551–558, 1998)

THE ADIPOCYTE hormone, leptin is implicated in the regulation of food intake, energy expenditure, and body fat stores (1). Circulating leptin decreases after fasting or caloric restriction in both humans (2–4) and rodents (5–7), and increases a number of hours after refeeding (3, 6). In humans, there is a nocturnal rise of plasma leptin (8), which has been hypothesized to be due to a delayed effect of insulin released during earlier meals. Consistent with this hypothesis, insulin increases expression of the *ob* gene in rodents (9–11) and in adipocytes *in vitro* (12, 13) after a number of hours. In humans, plasma insulin and leptin concentrations decrease in parallel after weight loss, independently of changes of adiposity (14). Furthermore, plasma leptin is negatively correlated with insulin sensitivity independently of adiposity in subjects with impaired glucose tolerance (15).

Short term insulin administration does not affect plasma leptin concentrations in human subjects (16, 17), but increases in circulating leptin have been reported after 4–6 h of high dose insulin administration (18, 19). These studies by necessity require the infusion of large amounts of glucose to prevent hypoglycemia. Similarly, prolonged hyperglycemia in response to extended glucose infusions increases plasma leptin after several hours in nonhuman primates (20) and human subjects (21); however, glucose administration also markedly increases endogenous insulin levels. Therefore, the role of insulin *per se* on the adipocyte *vs.* the effect of insulin to increase glucose flux into adipocytes was not addressed by these experiments.

Several lines of evidence have led us to hypothesize that glucose is an important regulator of leptin expression and secretion. First, increases in *ob* messenger RNA (mRNA) after glucose administration in mice are more closely related to plasma glucose concentrations than to plasma insulin concentrations (22). Second, infusion of small amounts of glucose to prevent the decline of glycemia during fasting in humans also prevents the decrease in plasma leptin (2). Third, the decrease in plasma leptin during marked caloric restriction in humans is better correlated with the decrease in plasma glucose than with changes in insulinemia (4). Fourth, we have found that low plasma leptin levels in streptozotocin diabetic rats are acutely increased by insulin administration in proportion to the degree of glucose lowering (23). Lastly, lowering plasma glucose concentrations in hyperglycemic insulin-dependent diabetic human subjects by

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infusing insulin at rates that produced physiological insulinemia increases circulating leptin (24).

To investigate the mechanisms by which glucose influences leptin secretion, we adapted and modified an *in vitro* system for culturing rat adipocytes in which the adipocytes are anchored in a defined mixture of extracellular matrix components (25) This matrix, Matrigel, appears to simulate normal basement membrane attachment of cells and may allow cell to cell interactions between adipocytes. Cells cultured in this system are, therefore, in an environment closer to their normal physiological milieu than in systems where adipocytes are free floating in the culture medium. Adipocytes cultured on Matrigel have been shown to maintain many of their differentiated characteristics and, in contrast with free-floating adipocytes, show no sign of dedifferentiation after 6 days of culture (25, 26). With this system we have investigated the regulation of leptin secretion by glucose and insulin and the effects of inhibitors of adipocyte glucose transport and metabolism on leptin secretion. The leptin (*ob*) mRNA content of the adipocytes after culture with insulin and inhibitors was also examined.

Materials and Methods

Materials

DMEM and FBS were purchased from Life Technologies (Grand Island, NY). The media were supplemented with 6 ml each of MEM nonessential amino acids, penicillin/streptomycin (5000 U/ml/5000 μg/ml), and nystatin (10,000 U/ml; all from Life Technologies) per 500 ml DMEM. BSA fraction V, HEPES, collagenase (*Clostridium histolyticum*; type II, Sigma Chemical Co., St. Lousi, MO; SA, 456 U/mg), insulin, D-glucose, sodium fluoride (NaFl), phloretin, iodoacetate, and fructose were purchased from Sigma Chemical Co. Matrigel matrix was purchased from Becton Dickinson (Franklin Lakes, NJ). 2-Deoxy-D-glucose (2-DG) was obtained from U.S. Biochemical Corp. (Cleveland, OH). Six-well Falcon tissue culture plates were purchased from Fisher Scientific (Pittsburgh, PA). Nylon filters were purchased from Tetko (Kansas City, MO).

Animals

Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Animals were housed in hanging wire cages in temperature controlled rooms (22–24 C) with a 12-h light-dark cycle and fed Purina chow diet (Ralston-Purina, St. Louis, MO) and given deionized water *ad libitum*. The study protocol was approved by the University of California-Davis animal care and use committee.

Cell isolation/preparation

Adipocytes were prepared from epididymal fat pads of male Sprague-Dawley rats (300–600 g) anesthetized with halothane. Epididymal fat depots were resected under aseptic conditions, and adipocytes were isolated by collagenase digestion according to the Rodbell procedure (27) with minor modifications as described below. The fat pads were minced into pieces in Krebs-Ringer HEPES buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2.2H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4, and 10 mM HEPES). Adipose tissue fragments were digested in the same buffer in the presence of type II collagenase (2.5 mg/2 ml buffer·g tissue) at 37 C with gentle shaking at 60 cycles/min for 45 min. The resulting cell suspension was diluted in 24 ml cold HEPES-phosphate buffer. Isolated adipocytes were separated from undigested tissue by filtration through a 400-μm nylon mesh and washed three times. For washing, cells were centrifuged at 500 rpm for 5 min. Each time the infranatant was discarded, and the adipocytes were resuspended in Krebs-Ringer HEPES buffer, with the final wash being in 0, 5, or 10 mM glucose culture medium supplemented with 1% or 5% FBS. The isolated adipocytes were then incubated for 30 min at 37 C before being plated in Matrigel-coated culture plates.

Adipocyte culture

Matrigel was thawed on ice to a liquid and uniformly applied to the surface of the culture dish (300 μl Matrigel/35-mm well). After the incubation, 150 μl of the adipocyte suspension (2:1 ratio of packed cells to medium) were plated on the liquid matrix. The warmth of the cells and buffer caused the Matrigel to gel around the adipocytes, effectively anchoring them to the culture dish. After a 30-min incubation at 37 C, 2 ml warm culture medium supplemented with FBS were added. The cells were maintained in an incubator at 37 C in 6% CO_2 for 96 h.

The initial medium concentration of glucose for the cultures conducted in the insulin dose-response experiment was 10.0–10.5 mM (180–190 mg/dl) to ensure that the cells would not deplete the glucose supply during the 96-h incubation when higher concentrations of insulin were used. Only 1% FBS was used in the insulin dose-response study to minimize the small amount of insulin present in the serum, which at 1% was less than 0.1 μU/ml. In the fructose study, medium made with glucose-free DMEM and 1% fetal serum was used to minimize the amount of glucose available to the adipocytes (<0.1 mmol/liter). However, it was not possible to eliminate all glucose from culture preparation because the Matrigel matrix itself contains ~4.2 mmol/liter glucose. For the fructose experiment, the Matrigel was diluted 1:2 with glucose-free medium to approximately 1.5 mmol/liter glucose.

In the other experiments with inhibitors of glucose transport, 2-DG (28), phloretin (29), and cytochalasin B (30), or with inhibitors of glycolysis, iodoacetate (31), and NaFl (32), the initial medium glucose concentration was (5.0–5.5 mM; 90–100 mg/dl) with 5% fetal serum. These agents were used at concentrations at or below those typically employed to inhibit glucose transport or glycolysis in adipocytes (28–32). Cytochalasin B was initially dissolved in ethanol and diluted to 0.5% ethanol in the well with the highest dose. Therefore, the medium in all wells in the cytochalasin B experiment was equalized to 0.5% ethanol. Aliquots of adipocytes from each animal were divided into wells with the responses to insulin, the various inhibitors, or fructose being compared with those of an appropriate control well containing adipocytes from the same animal. In a preliminary insulin dose-response study, we found that medium leptin concentrations in the presence of insulin were not increased over those in control medium (no insulin) until after 24 h of incubation. Therefore, for the remainder of the studies, 300-μl samples (15% of the medium volume) were collected at 24, 48, 72, and 96 h and replaced with 300 μl fresh medium containing the appropriate concentrations of glucose, insulin, and/or inhibitors. Cultures were observed daily with a phase contrast microscope. After 96 h, a subset of the culture plates was frozen until analyzed for leptin (*ob*) mRNA content by Northern blot.

Assays

Leptin concentrations in the medium were determined with a sensitive and specific RIA for mouse leptin as previously described (7) (Linco Research, St. Charles, MO). Leptin concentrations in medium from cultured rat adipocytes measured with this assay are very similar to those obtained with a newly developed assay specific for rat leptin. With the rat-specific assay, measured leptin concentrations in culture medium were 86 ± 3% of the mouse values and were highly correlated between the two assays ($r = 0.97$; $P < 0.0001$; unpublished data). Therefore, measurements of rat leptin made with the mouse assay provide a reliable measurement of leptin concentrations. The intra- and interassay coefficients of variation for this assay are 4.0% and 11.2%, respectively (7). The antibody used in the assay does not cross-react with insulin, proinsulin, glucagon, pancreatic polypeptide or somatostatin. Glucose and lactate were measured with a YSI glucose analyzer (model 2300, Yellow Springs Instruments, Yellow Springs, OH).

Northern blot procedure

The following procedures were performed on culture plates incubated with 5 mM glucose and 5% fetal serum alone (control), 1.6 nM insulin, and 1.6 nM insulin with 10 mg/dl 2-DG or 1 mM NaFl for 48 and 96 h. Northern blot analysis was performed as previously described (33).

In brief, 1 ml RNAzol B (Tel-Test, Friendswood, TX) was added directly to the wells containing the adipocytes and matrix. The solution was repetitively taken in and expelled from the pipette to maximize dissolution of the adipose tissue. UV absorbance and integrity gels were used to estimate RNA. To allow loading of equal mass of RNA in each well, after analysis of leptin mRNA using a single-stranded complementary DNA probe followed by quantification of bands on a phosphoimager as well as from film, the blots were reanalyzed using a probe complementary to mouse 18S ribosomal RNA. Leptin mRNA was then normalized with respect to the 18S ribosomal signal, according to the absolute signal. The 18S RNA results were virtually identical in all cases. In particular, experimental conditions did not influence the 18S ribosomal signal.

Calculations and data analysis

The uptake of glucose was assessed by measuring the concentration of glucose in the medium in each well before and after 96 h of incubation and calculating the decrease over 96 h. To examine the relationship between adipocyte carbon flux and leptin secretion in response to increased insulin-mediated glucose uptake, the amount of carbon released as lactate per amount of carbon taken up as glucose over 96 h was calculated as Δ[lactate]/Δ[glucose], where Δ is the change, and expressed as a percentage. The area under the curve for leptin concentrations in the medium between 0–96 h was calculated by the trapezoidal method. The means of two groups were compared by paired *t* test. The means of more than two groups were compared by ANOVA. To examine the relationships between the medium concentrations of insulin or inhibitors employed, the amount of glucose taken up by the adipocytes, and leptin secretion, simple and multiple linear regression analyses were performed with a statistics software package (StatView for Macintosh, Abacus Concepts, Inc., Berkeley, CA). Data are expressed as the mean ± SEM.

Results

Responses to insulin (0.16–16.0 nM)

The effects of insulin on leptin secretion, and the relationship between glucose uptake by adipocytes cultured with different concentrations of insulin and leptin secretion were examined. Insulin produced a concentration-dependent increase in glucose uptake by the cultured adipocytes ($r = 0.61$; $P < 0.0002$ *vs.* insulin concentration), as assessed by the decrease in glucose in the medium (Fig. 1A). With no added insulin, the medium glucose concentration decreased from 10.1 ± 0.1 to 8.2 ± 0.3 mmol/liter (Δ, −1.9 ± 0.3 mmol/liter; $P < 0.0001$). The addition of 0.16, 1.6, and 16.0 nM insulin increased glucose uptake (Δglucose, −2.7 ± 0.4, −3.3 ± 0.3, and −3.9 ± 0.4 mmol/liter, respectively; all $P < 0.01$ *vs.* no insulin). Insulin also produced a concentration-dependent increase in lactate production ($r = 0.70$; $P < 0.0001$), which was well correlated with the decrease in glucose in the medium over 96 h ($r = 0.61$; $P < 0.0002$), suggesting that a significant portion of the glucose entering the adipocytes was metabolized only as far as lactate and released from the cells into the medium (34, 35).

Leptin secretion was increased over the control value by all three concentrations of insulin (Fig. 1B). The production of lactate was not related to the leptin response ($r = 0.10$; $P = 0.59$). The area under the leptin concentration curve (AUC) from 0–96 h was independently related to the decrease in glucose in the medium during the incubation (Fig. 1C), but not to the insulin concentration (Table 1). Similarly, with a multiple regression model, the AUC for leptin was related to the decrease in glucose, but not to the insulin concentration. In addition, the percentage of carbon released as lactate per amount of carbon taken up as glucose was calculated. Over-

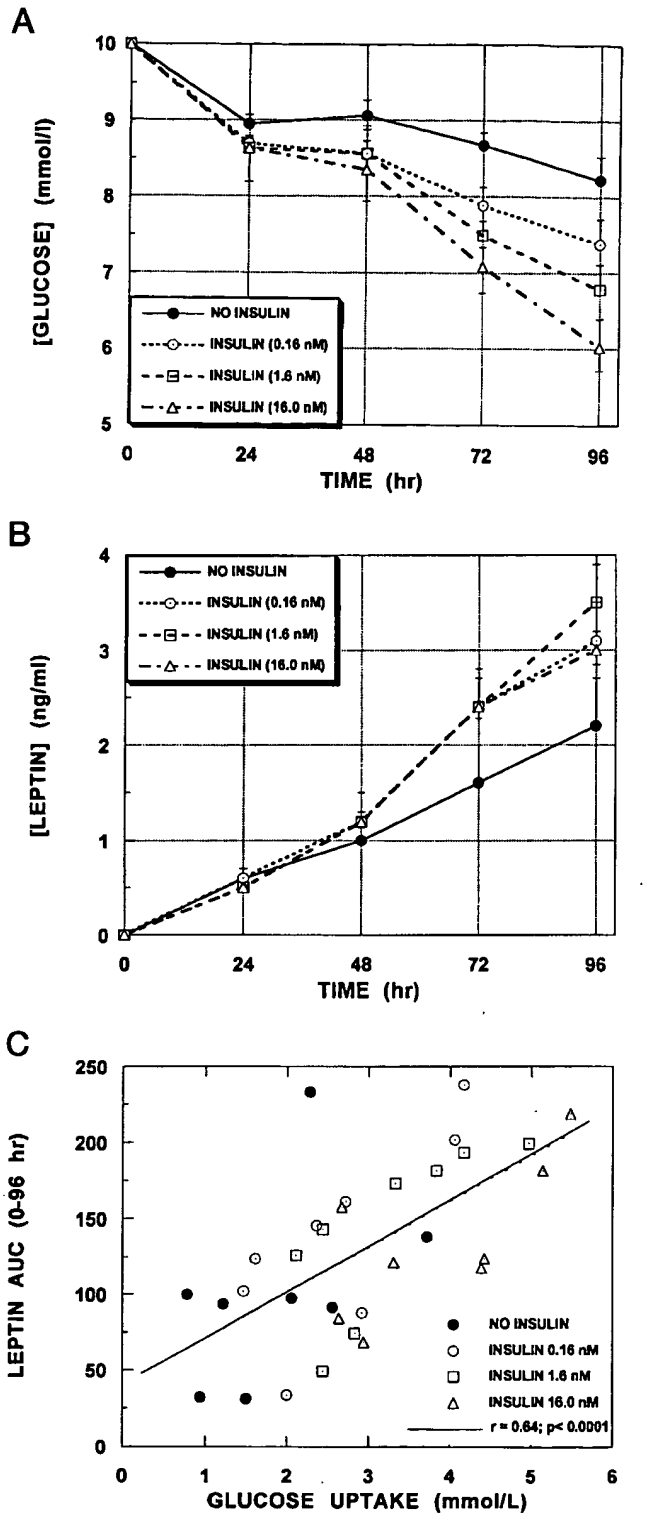

FIG. 1. A, Glucose concentrations in medium from 0–96 h from isolated rat adipocytes in primary culture with insulin concentrations from 0–16 nM (n = 8/treatment). B, Leptin concentrations from 0–96 h from isolated rat adipocytes in primary culture with insulin concentrations from 0–16 nM (n = 8/treatment). C, Relationship between glucose uptake, as assessed by the decrease in glucose in the culture medium, and leptin secretion, expressed as the AUC from 0–96 h, during incubation of adipocytes with 0–16 nM insulin (n = 32).

TABLE 1. Relationship between glucose uptake (ΔGluc), leptin secretion (ΔLept) and the concentration of insulin or inhibitors of glucose uptake and metabolism after 96-h incubation of adipocytes with insulin and insulin plus inhibitors (2-DG, phloretin, cytochalasin-B, iodocetate, or sodium fluoride at varying concentrations; see *Materials and Methods*)

Insulin or inhibitor (n)	Simple Regression				Multiple regression	
	ΔLept *vs.* ΔGluc		ΔLept *vs.* [inhibitor]		ΔLept *vs.* ΔGluc (*P*)	ΔLept *vs.* [inhibitor] (*P*)
	r	*P*	r	*P*		
Insulin (32)	0.64	0.0001	0.20	0.28	0.0001	0.09
2-DG (38)	0.67	0.0001	0.51	0.001	0.001	0.29
Phloretin (38)	0.86	0.0001	0.78	0.0001	0.0012	0.75
Cytochalasin-B (19)	0.60	0.01	0.58	0.02	0.22	0.25
Iodoacetate (34)	0.83	0.0001	0.74	0.0001	0.0001	0.17
Sodium fluoride (28)	0.85	0.0001	0.60	0.001	0.0001	0.73

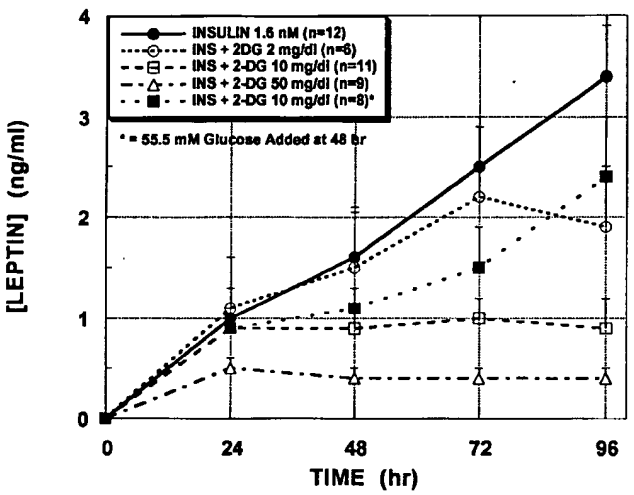

FIG. 2. Effects of inhibiting glucose transport and metabolism with 2-DG on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin and the effect of adding glucose (55 mM) at 48 h on the inhibition of leptin secretion produced by 10 mg/dl 2-DG.

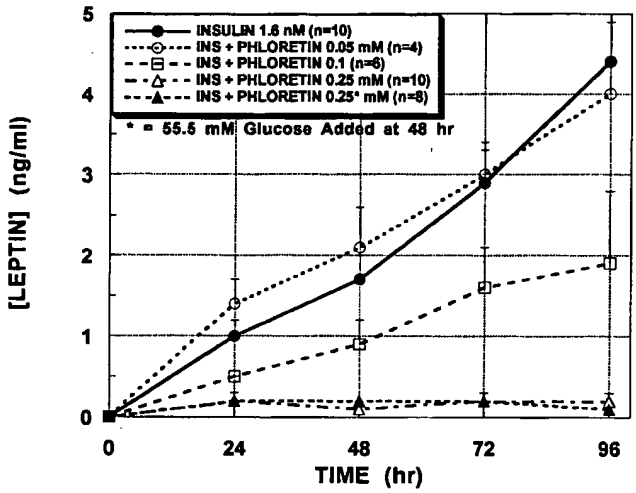

FIG. 3. Effects of inhibiting glucose transport with phloretin on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin and the effect of adding glucose (55 mM) at 48 h on the inhibition of leptin secretion produced by 0.25 mM phloretin.

all, in the insulin experiment between 10–68% of the amount of carbon taken up as glucose was released as lactate (mean, 34 ± 2%). There was no direct relationship between this parameter and the insulin concentration; however, it was inversely proportional to the amount of leptin secreted, as expressed by the 0–96 h leptin AUC ($r = 0.64$; $P < 0.0001$). By multiple regression analysis, the relationship between glucose conversion to lactate and leptin secretion was not significantly related to lactate production ($P = 0.06$), but leptin secretion was equally related to both the change in glucose and the amount of glucose carbon released as lactate (both $P < 0.001$).

Effects of 2-DG (2–50 mg/dl)

The effect of inhibiting glucose uptake and metabolism with 2-DG on leptin secretion and its relationship to adipocyte glucose uptake were examined. 2-DG at a concentration of 50 mg/dl completely inhibited glucose uptake (Δ, 0.1 ± 0.3 mmol/liter) in the presence of 1.6 nM insulin (Δglucose, −4.0 ± 0.6 mmol/liter) and inhibited the leptin response (AUC 0–96 h) by 69 ± 4% ($P < 0.0001$) compared with insulin alone (Fig. 2A). At a lower concentration of 2-DG (10 mg/dl), glucose uptake was still markedly inhibited (Δ, −0.1 ± 0.4 mmol/liter) and leptin secretion was inhibited by 47 ± 5% ($P < 0.0001$). The lowest concentration of 2-DG (2 mg/dl) produced less of an inhibition of glucose uptake (Δ, −1.5 ± 0.9 mmol/liter; $P < 0.01$ *vs.* insulin alone). At this concentration, the leptin response was not significantly inhibited until the 96 h point ($P < 0.02$ *vs.* insulin alone; Fig. 2A).

Overall, the change in leptin at 96 h was related to the concentration of 2-DG and was well correlated with the decrease in medium glucose (Table 1). By multiple regression, the leptin concentration in the medium at 96 h was significantly correlated with the change in glucose, but not to the 2-DG concentration (Table 1). The addition of glucose (55.5 mM) at 48 h reversed the inhibition of leptin secretion produced by 2-DG at 10 mg/dl by 96 h ($P < 0.01$ *vs.* 2-DG; NS *vs.* insulin alone; Fig. 2).

Effects of phloretin (0.05–0.25 mM)

The effect of inhibiting glucose uptake with phloretin on leptin secretion was examined. Phloretin at a concentration of 0.25 mM completely inhibited leptin secretion (Fig. 3). The 0–96 h AUC for leptin was inhibited by 91 ± 2% of insulin alone ($P < 0.0001$). This higher concentration of phloretin (0.25 mM) also completely blocked glucose uptake in the

presence of 1.6 nM insulin (Δglucose, 0.7 ± 0.1 mmol/liter). Overall, the leptin response was inversely related to the concentration of phloretin and was highly correlated with the decrease in glucose in the medium (Table 1). However, by multiple regression, the leptin response was correlated with the decrease in glucose, but not with the concentration of phloretin (Table 1). The addition of 55.5 mM glucose at 48 h did not reverse the inhibition of leptin secretion by phloretin (Fig. 3).

Effects of cytochalasin B

The effect of inhibiting glucose uptake with cytochalasin B on leptin secretion was examined. Cytochalasin B produced a concentration-dependent inhibition of glucose uptake and leptin secretion (Fig. 4). The leptin response was significantly correlated with glucose uptake by simple regression (Table 1), but was not significantly correlated with glucose uptake (as observed with the other inhibitors; Table 1) by multiple regression, perhaps due to the smaller number of replicates (n = 19) in this experiment.

Effects of iodoacetate (0.005–1.0 mM)

The effect of inhibiting glycolysis with iodoacetate on leptin secretion was examined. Iodoacetate at 1.0, 0.1, and 0.01 mM markedly inhibited glucose uptake (Δglucose, −0.1 ± 1.1, 0.5 ± 0.2, and 0.3 ± 0.2 mmol/liter, respectively) and leptin secretion. The 0–96 h AUC for leptin was inhibited by −95 ± 2%, −91 ± 2%, and −87 ± 3%, respectively, compared with insulin alone; (all $P < 0.0001$). The lowest concentration of iodoacetate (0.005 mM) produced less of an inhibition of glucose uptake (Δglucose, −1.8 ± 0.8 mmol/liter) and less of an inhibition of leptin secretion (−51.0 ± 16%) than insulin alone ($P < 0.02$; Fig. 5). By simple regression, the release of leptin was related to the concentration of iodoacetate and was highly correlated with the change in glucose in the medium (Table 1). However, by multiple regression, the leptin secreted at 96 h was related to the change in glucose, but not to the concentration of iodoacetate (Table 1).

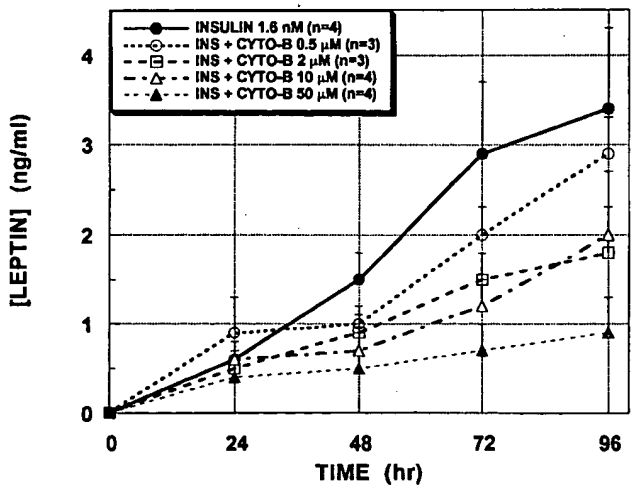

FIG. 4. Effects of inhibiting glucose transport with cytochalasin B on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

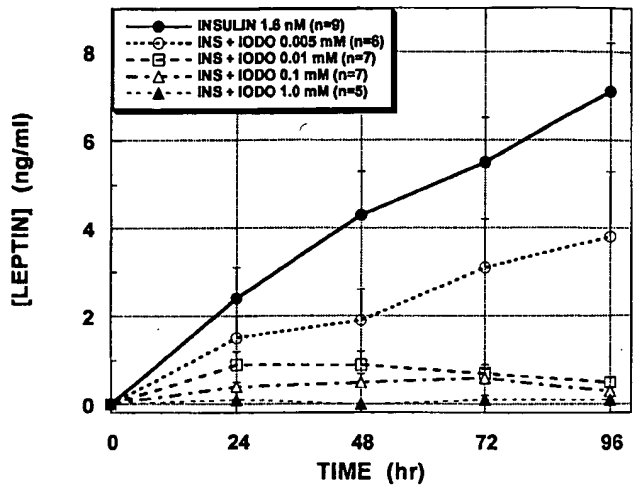

FIG. 5. Effects of inhibiting glycolysis with iodoacetate on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

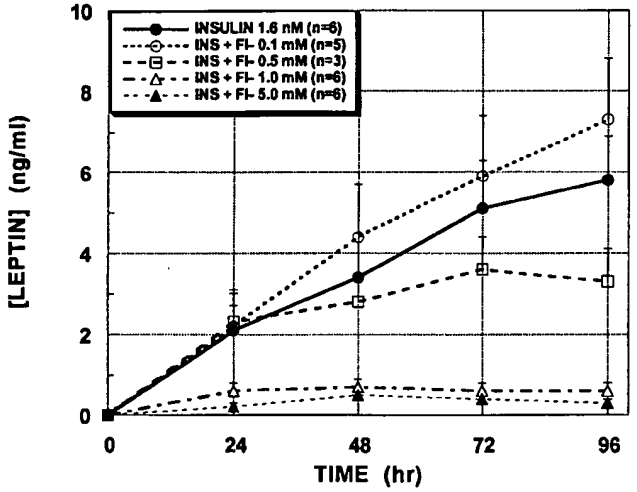

FIG. 6. Effects of inhibiting glycolysis with NaFl on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture for 96 h with 1.6 nM insulin.

Effects of NaFl (0.1–5.0 mM)

The effect of inhibiting glycolysis with NaFl was examined. The two highest concentrations of NaFl (5.0 and 1.0 mM) completely inhibited glucose uptake (Δglucose, 0.2 ± 0.1 and 0.0 ± 0.3 mmol/liter, respectively). The 0.5 mM concentration of NaFl produced less of an inhibition of glucose uptake (Δ, −2.1 ± 0.6 mmol/liter), and the lowest concentration (0.1 mM) of NaFl did not inhibit glucose uptake (Δglucose, −3.9 ± 0.5 mmol/liter) compared with the effect of insulin alone. The two highest concentrations of NaFl (5.0 and 1.0 mM) markedly inhibited leptin secretion (−81 ± 6% *vs.* insulin alone; $P < 0.0001$). The next concentration of NaFl (0.5 mM) produced an intermediate inhibition of leptin secretion (−47 ± 15% of insulin alone; $P < 0.05$). The 0.1-mM concentration of NaFl did not inhibit leptin secretion (−4 ± 15% *vs.* insulin alone; P = NS; Fig. 6).

Overall, the decline in medium glucose was significantly

correlated with the concentration of NaFl and highly correlated with the 96 h leptin concentration (Table 1). By multiple regression, the amount of leptin secreted at 96 h was strongly correlated with the change in glucose in the medium ($P < 0.0001$), but not to the NaFl concentration (Table 1).

Effects of insulin, 2-DG, and NaFl on leptin (ob) mRNA and 18S ribosomal RNA

The effects of inhibiting glucose uptake and metabolism with 2-DG or NaFl on leptin gene expression and ribosomal 18S RNA were examined. As shown in Fig. 7A, leptin (*ob*) mRNA was detectable in adipocytes incubated for 48 h either with 1.6 nM insulin or without insulin (control). However, the leptin mRNA signal was reduced to near undetectable levels when adipocytes were incubated with 1.6 nM insulin and either 2-DG (10 mg/ml) or 1.0 mM NaFl (Fig. 7A). The effect of 2-DG and NaFl was specific, because in the same samples there was no effect of these concentrations of 2-DG or NaFl on 18S ribosomal RNA (Fig. 7B) or on nonspecific RNA bands (with a different mol wt than leptin mRNA) that could be detected on the Northern blots after long exposures (data not shown). Leptin mRNA was significantly reduced by 2-DG or NaFl regardless of whether the signal was normalized for 18S ribosomal signal ($P = 0.0174$). Qualitatively similar effects of 2-DG or NaFl were observed in cultures incubated for 96 h ($P = 0.0228$; data not shown).

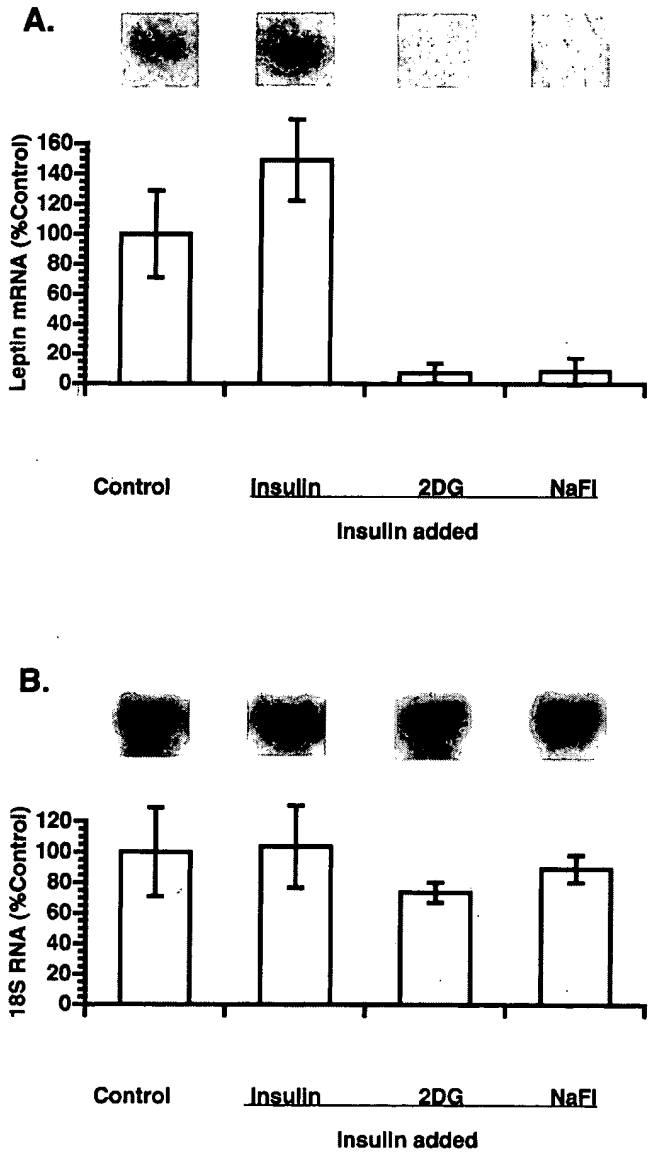

FIG. 7. A, Effects of no insulin (control), 1.6 nM insulin, and 1.6 nM insulin plus 10 mg/dl 2-DG or 1.0 mM NaFl on leptin (*ob*) mRNA after 48 h of incubation, as assessed by Northern blots. The *inset above each bar* is representative of the signal obtained for each condition. B, Effects of control (no insulin), 1.6 nM insulin, and 1.6 nM insulin plus 10 mg/dl 2-DG or 1.0 mM NaFl on 18S ribosomal RNA after 48 h of incubation, as assessed by Northern blots. The *inset above each bar* is representative of the signal obtained for each condition.

Effects of fructose (5 mM)

The addition of 5 mM fructose to medium of cultures in which the glucose concentration was minimized by diluting the Matrigel 1:2 and using glucose-free DMEM with 1% serum augmented leptin secretion after 48 h. The initial response in the control wells was probably due to the residual glucose (~1.5 mmol/L) in the diluted Matrigel. However, both the integrated AUC from 0–96 h ($P < 0.02$) and the leptin concentration at 96 h ($P < 0.01$) were increased by fructose (Fig. 8).

Discussion

In the present study we found that addition of physiological concentrations of insulin stimulates leptin secretion from isolated rat adipocytes in primary culture. In this *in vitro* system we did not see an acute effect of insulin on leptin secretion. This is in agreement with previous reports that have demonstrated that the expression of *ob* gene and leptin protein release are not acutely regulated by insulin *in vivo* and *in vitro* (17, 36). The strong correlation between adipocyte glucose uptake measured by the decrease in glucose in the media during incubation with insulin and the amount of

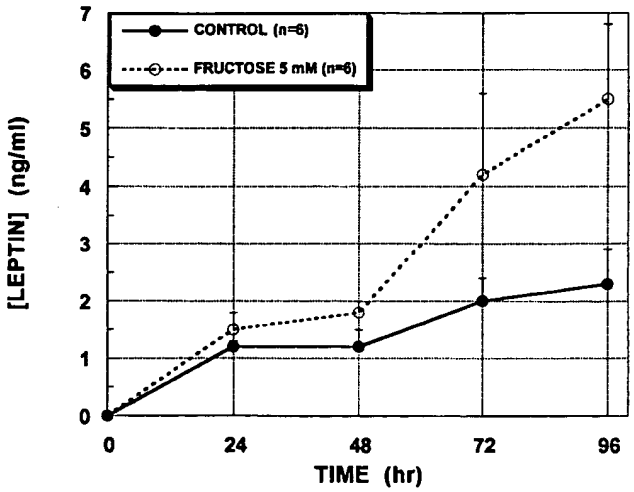

FIG. 8. Effect of fructose (5 mM) on leptin concentrations from 0–96 h in medium from isolated rat adipocytes in primary culture with a low (~1.5 mmol/liter) initial glucose concentration (n = 6/treatment).

leptin secreted from isolated adipocytes is consistent with the hypothesis that the rate of glucose metabolism is a determinant of leptin secretion. In addition, the absolute insulin concentration was not related to the leptin response, independently of the effect of insulin to increase glucose uptake.

Blockade of glucose transport with 2-DG, phloretin, or cytochalasin B at concentrations at or below those typically used in adipocytes (29, 30) produced a dose-dependent decrease in leptin secretion in the presence of high physiological concentrations of insulin. The competitive inhibition produced by 2-DG could be reversed by the addition of a high concentration of glucose, suggesting that 2-DG did not inhibit leptin secretion via a nonspecific toxic effect on the adipocytes. As expected, the inhibition by phloretin was not reversed by glucose, as phloretin is not a competitive inhibitor and, therefore, produces an irreversible inhibition of glucose transport that is not readily overcome by high glucose concentrations. These experiments provide evidence that glucose uptake is required to increase leptin secretion from isolated adipocytes despite the presence of high physiological insulin concentrations.

Inhibition of glycolysis with either iodoacetate or NaFl at low concentrations (31, 32) also produced concentration-dependent inhibition of leptin secretion in the presence of insulin. When glycolysis is inhibited, glycolytic intermediates accumulate, resulting in a secondary impairment of glucose uptake. As with primary blockade of glucose uptake, during inhibition of glucose metabolism by either glycolytic inhibitor, the amount of glucose taken up over 96 h of incubation was highly correlated with the amount of leptin secreted despite the presence of insulin. These results suggest that the stimulation of leptin secretion by insulin is unlikely to be due to a direct effect of insulin *per se*, but is secondary to the effect of insulin to stimulate glucose uptake and metabolism in adipocytes.

We also found that inhibition of glucose transport and metabolism with 2-DG or glycolysis with NaFl markedly inhibited leptin (*ob*) gene expression, as assessed by Northern blot analysis of leptin mRNA. In the same cultures, 18S ribosomal RNA levels were unaffected by either 2-DG or NaFl, suggesting that the decrease in leptin gene expression was not due to a nonspecific overall effect of these inhibitors to impair adipocyte RNA synthesis. In addition, we examined the amount of heparin-released lipoprotein lipase (LPL) from adipocytes cultured with the various inhibitors (data not presented). Although LPL was modestly decreased by the inhibitors (~25–50% of insulin-stimulated levels), the suppression of leptin secretion was significantly greater (80–90%), suggesting a relative specificity of blocking glucose uptake and metabolism on leptin secretion *vs.* that on another protein (LPL) produced by adipose tissue. Lastly, the effects of the blockers to inhibit leptin expression and secretion are unlikely to be due to a depletion of adipocyte energy stores, as it is known that adipocytes can generate energy (ATP) by oxidizing fatty acids via mitochondrial β-oxidation (37, 38).

Taken together, these data suggest a physiological role for glucose in the regulation of leptin expression and secretion by adipocytes. Accordingly, we hypothesize that during fasting, when circulating insulin and glucose concentrations are low and glucocorticoids are elevated, leptin secretion declines secondary to decreased glucose transport into adipose tissue. Upon refeeding, increases in circulating insulin and glucose and the resulting increases in adipose glucose uptake and metabolism stimulate leptin secretion and restores circulating leptin concentrations to prefasting levels. This model, therefore, can explain the effects of fasting and refeeding on circulating leptin in humans (2–4) and rodents (5–7). In addition, the nocturnal increase in plasma leptin observed in humans could potentially arise as a delayed consequence of increased insulin-stimulated glucose metabolism following meals (8). The effect of glucose infusions to prevent the fall of plasma leptin during fasting in human subjects may be similarly mediated (2).

Thus, leptin secretion appears to reflect the amount of glucose transported and metabolized by adipose tissue. There is convincing evidence that suggests that a significant portion of glucose entering adipose tissue is metabolized to lactate and released (34, 35). This lactate may contribute to the pool of gluconeogenic precursors during fasting. Our results show that when a smaller proportion of glucose carbon taken up by adipocytes is released as lactate, more leptin is secreted. These data are consistent with the changes in leptin secretion observed during fasting and refeeding. In addition, fructose, in the presence of low glucose concentrations, stimulates leptin secretion, demonstrating that a non-glucose substrate can induce the adipocyte to secrete leptin and suggesting that stimulation of leptin secretion by glucose metabolism occurs downstream of phosphofructokinase.

In summary, blockade of glucose transport or inhibition of glycolysis inhibits leptin secretion from and gene expression in isolated cultured adipocytes. The secretion of leptin is directly proportional to the amount of glucose taken up by the adipocytes. These results suggest that leptin secretion is linked to glucose transport and metabolism and help to explain the known effects of feeding/fasting and long term glucose and insulin administration on circulating leptin concentrations.

Acknowledgments

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Effects of Metformin and Vanadium on Leptin Secretion from Cultured Rat Adipocytes

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Abstract

MUELLER, WENDY M., KIMBER L. STANHOPE, FRANCINE GREGOIRE, JOSEPH L. EVANS, AND PETER J. HAVEL. Effects of metformin and vanadium on leptin secretion from cultured rat adipocytes. *Obes Res.* 2000;8:530–539.

Objective: We have reported that glucose utilization regulates leptin expression and secretion from isolated rat adipocytes. In this study, we employed two antidiabetic agents that act to increase glucose uptake by peripheral tissues, metformin and vanadium, as pharmacological tools to examine the effects of altering glucose utilization on leptin secretion in primary cultures of rat adipocytes.

Research Methods and Procedures: Isolated adipocytes (100 μL of packed cells per well) were anchored in a defined matrix of basement membrane components (Matrigel) with media containing 5.5 mM glucose and incubated for 96 hours with metformin or vanadium. Leptin secretion, glucose utilization, and lactate production were assessed.

Results: Metformin (0.5 and 1.0 mM) increased glucose uptake in the presence of 0.16 nM insulin by 37 ± 10% ($p < 0.005$) and 62 ± 8% ($p < 0.0001$) over insulin alone, respectively. Metformin from 0.5 to 5.0 mM increased lactate production by 105 ± 43% ($p < 0.025$) to 202 ± 52% ($p < 0.0025$) and at 1.0 and 5.0 mM increased the proportional rate of glucose conversion to lactate by 78 ± 18% ($p < 0.005$) and 166 ± 41% ($p < 0.0025$), respectively. At concentrations less than 0.5 mM, metformin did not affect leptin secretion, but at 0.5 mM, the only concentration that significantly increased glucose utilization without increasing glucose conversion to lactate, leptin secretion was modestly stimulated (by 20 ± 9%; $p < 0.05$). Concentrations from 1.0 to 25 mM inhibited leptin secretion by 25 ± 8% ($p < 0.005$) to 89 ± 4% ($p < 0.0001$). Across metformin doses, leptin secretion was inversely related to the percentage of glucose taken up and released as lactate ($r = -0.74$; $p < 0.0001$). Vanadium (5 to 20 μM) increased glucose uptake from 20 ± 7% ($p < 0.01$) to 34 ± 13% ($p < 0.02$) and increased lactate production at 5 μM by 17 ± 8% ($p < 0.025$) and 10 μM by 61 ± 20% ($p < 0.02$) but did not alter the conversion of glucose to lactate. Vanadium (5 to 50 μM) inhibited leptin secretion by 33 ± 6% ($p < 0.0025$) to 61 ± 8% ($p < 0.0001$).

Discussion: Both metformin and vanadium increase glucose uptake and inhibit leptin secretion from cultured adipocytes. The inhibition of leptin secretion by metformin is related to an increase in the metabolism of glucose to lactate. The inhibition by vanadium most likely involves direct effects on cellular phosphatases. We hypothesize that the effect of glucose utilization to stimulate leptin production involves the metabolism of glucose to a fate other than anaerobic lactate production, possibly oxidation or lipogenesis.

Key words: glucose uptake, lactate production, anaerobic metabolism

Introduction

The adipocyte hormone, leptin, has a central role in the regulation of food intake, energy expenditure, and body fat stores (1,2). Circulating leptin concentrations are well correlated with adipose stores in humans (3–5) and animals (5–7). However, leptin production is also acutely regulated by nutritional status. For example, circulating leptin decreases after fasting (6,8–10) or energy restriction (11,12) and increases after refeeding or overfeeding (9,13). These changes of circulating leptin are disproportionate to the relatively small changes of body fat. Although the regulation of leptin expression and secretion is incompletely understood, changes of insulin secretion during fasting and refeeding precede changes of circulating leptin concentrations. There is a growing body of evidence that suggests a role for insulin and glucose in mediating changes of circulating leptin levels in vivo. For example, infusion of small amounts of glucose to prevent the reductions of insulin and

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glucose during fasting in humans also prevents the decrease in plasma leptin (8). Although insulin administration does not acutely increase plasma leptin concentrations in human subjects (14,15) increases have been reported after 4 to 6 hours during insulin infusions producing supraphysiological (16,17) or physiological (18) increments of plasma insulin levels. Similarly, prolonged hyperglycemia and hyperinsulinemia in response to extended glucose infusions increases plasma leptin after several hours in nonhuman primates (7) and human subjects (19). Lastly, leptin concentrations increase 4 to 6 hours after high carbohydrate meals, which induce large plasma insulin and glucose responses in humans (20).

In vitro studies have shown that insulin increases leptin expression and secretion in isolated rodent (21–23) and human (15,24) adipocytes. It has not, however, been clear whether the effect of insulin to increase leptin production is a direct consequence of increased insulin signaling or might be indirectly mediated by insulin's actions on glucose metabolism. Several in vivo studies have provided support for the latter explanation. First, glucose administration induces increases of *ob* mRNA expression, which are more closely related to changes of plasma glucose than to plasma insulin concentrations (25,26).

In addition, the decrease in plasma leptin during marked caloric restriction in humans is better correlated with the decrease in plasma glucose than with changes in insulinemia (11,12). Furthermore, we have found that low plasma leptin levels in insulin-deficient streptozotocin diabetic rats are acutely increased by insulin administration in proportion to the degree of glucose lowering (27). Further support from in vitro experiments for a role for adipose glucose utilization in the regulation of leptin production is provided by our recent report that increased glucose metabolism is an important mediator of insulin-stimulated leptin expression and secretion. Blockade of glucose uptake or inhibition of glycolysis decreases *ob* gene expression and leptin secretion in isolated rat adipocytes (28). However, glucose uptake, by itself, only seems to be important in that glucose must first be taken up by the adipocytes before it can be metabolized. Rather than glucose uptake per se, the inverse relationship observed, between the proportional conversion of glucose to lactate and leptin secretion by isolated adipocytes (28), suggests that a regulatory step for glucose metabolism to mediate changes of leptin production involved the metabolism of glucose to a point beyond the anaerobic metabolism of glucose-derived pyruvate to lactate.

Metformin and vanadium are two antidiabetic agents, which are able to enhance glucose uptake and utilization by peripheral tissues (29,30). In the present study, we employed metformin and vanadium as pharmacological tools to examine the effects of altering adipocyte glucose utilization on leptin production in primary cultures of isolated adipocytes. Glucose utilization, lactate production, and leptin secretion were measured over 96 hours in isolated rat adipocytes cultured in a basement membrane matrix that maintains adipocyte differentiation.

Research Methods and Procedures

Materials

Media (Dulbecco's modified Eagle's medium [DMEM]) and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). The media was supplemented with 6 mL each of minimal essential medium amino acids, penicillin/streptomycin (5000 U/mL/5000 μg/mL), and nystatin (10,000 U/mL; all from Life Technologies) per 500 mL of DMEM. Bovine serum albumin fraction V, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), collagenase (*Clostridium histolyticum*, type II; specific activity, 456 U/mg), insulin, and metformin were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel matrix was purchased form Becton Dickinson (Franklin Lakes, NJ). Bis(maltolato) oxovanadium(IV) (BMOV), an organified form of vanadium (31), was a gift from Drs. John McNeill and Violet Yuen, Department of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, Canada. Six-well Falcon plates were purchased from Fisher Scientific (Pittsburgh, PA). Nylon filters were purchased from Tetko (Kansas City, MO).

Animals

Male Sprague-Dawley rats (3 to 6 months of age) were obtained from Charles River (Wilmington, MA). Animals were housed in hanging wire cages in temperature-controlled rooms (22 °C) with a 12-hour light-dark cycle and fed Purina chow diet (Ralston-Purina, St. Louise, MO) and given deionized water ad libitum. Animal use and care was in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals and conducted in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. The study protocol was approved by the Administrative Animal Use and Care Committee at University of California, Davis.

Methods

Cell Isolation/Preparation. Adipocytes were prepared from epididymal fat pads from male Sprague-Dawley rats weighing 300 to 600 g. Epididymal fat depots were resected from halothane-anesthetized rats under aseptic conditions, and adipocytes were isolated by collagenase digestion by the Rodbell method (32) with minor modifications as previously described (28). The isolated adipocytes were then incubated for 30 minutes at 37 °C before being plated and cultured on Matrigel-coated plates.

Adipocyte Culture. Adipocytes were maintained in culture anchored to a basement membrane matrix (Matrigel, Becton Dickinson). Although all in vitro systems have

inherent advantages and disadvantages, advantages of this system compared with cultures containing free-floating adipocytes are that the matrix simulates their normal basement membrane attachment and that the cells are maintained in close proximity to each other, allowing direct cell-to-cell contact. Together the cell contact and basement membrane attachment help to maintain differentiation, because adipocytes have a strong tendency to dedifferentiate in long term (>24-hour) culture. In addition, the matrix and the small amount of serum in the media both contain growth factors, which are also likely to help maintain cell differentiation. Furthermore, the adipocytes in this system are not exposed to toxic levels of oxygen at the interface of the media and the incubator atmosphere, as opposed to free-floating adipocytes which aggregate at the surface of the media. An advantage of the system over those containing minced adipose tissue is that all of the cells in the culture are equally exposed to the nutrients and the oxygen dissolved in the media. Thus, although clearly different from the in vivo situation, we believe that this system provides a more physiological environment than most systems for maintaining adipocytes in long term culture. In the case of the present studies, the goal was to examine the direct actions of metformin and vanadium on leptin production and adipocyte metabolism. Therefore, the advantage of employing in vitro experimentation for this purpose over in vivo models was that it was possible to control confounding variables, such as effects of these agents on food intake, which would indirectly influence leptin production via changes of insulin secretion (18,20). Unlike an in vivo system, in this study the environment surrounding the adipocytes within the individual wells of each culture plate was identical with the exception of the presence or absence and the concentration of metformin or vanadium, allowing assessment of the direct effects of the treatments.

In culturing each suspension, Matrigel was first thawed on ice to a liquid and uniformly applied to the surface of culture dishes (300 μL of Matrigel/35-mm well). After the incubation period, 150 μL of the adipocyte suspension (2:1 ratio of packed cells to media) were plated on the liquid Matrigel matrix. Adipocytes from each suspension were thoroughly mixed with a transfer pipette before plating to insure that a similar number of adipocytes with a similar size distribution were added to the control and experimental wells for each suspension. The warmth of the added cells and media caused the Matrigel to gel around the adipocytes, effectively anchoring them to the culture dish. After a 30-minute incubation at 37 °C, 2 mL of warm culture medium was added. The cells were maintained in an incubator at 37 °C for 96 hours with 6% CO_2. Aliquots of adipocytes from each animal were divided into wells, with the different concentrations of either metformin or vanadium (as detailed below). In each plate an appropriate control well contained adipocytes from the same animal. Adipocytes were incubated with media (DMEM) containing 5.5 mM (100 mg/dL) glucose plus 5% FBS at five concentrations of Metformin (0.1, 0.25, 0.5, 1.0, 5.0, and 25.0 mM). A low basal concentration of insulin (0.16 nM) was added to the incubations performed with metformin, because metformin is thought to act in part by increasing insulin signaling (33,34). Vanadium was added at four concentrations (5.0, 10.0, 20.0, and 50.0 μM) in DMEM with 5.0 to 5.5 mM glucose and 1% FBS. Adipocytes were cultured with vanadium without added insulin, because vanadium action is considered to be largely independent of insulin (35,36). To examine the responses to insulin in the adipocytes obtained from each adipocyte suspension in the vanadium experiments, a separate well containing 1.6 nM insulin was included for each suspension. In all experiments, aliquots of media (300 μL, 15% of the media volume) were collected from culture wells and replaced with fresh media containing the appropriate concentrations of metformin or vanadium at 24, 48, 72, and 96 hours.

Assays. Leptin concentrations in the medium were determined with a sensitive and specific radioimmunoassay for rat leptin (37) with reagents obtained from Linco Research, St. Charles, MO. Glucose and lactate were measured with a glucose analyzer (model 2300, YSI, Yellow Springs, OH).

Data Analysis. The uptake of glucose was assessed by measuring the concentration of glucose in the media in each well before and at 24, 48, 72, and 96 hours of incubation and calculating the decrease over 96 hours after correcting for the amount of glucose that was removed during each 24-hour media sampling and the amount added by the replacement of fresh media (15% of total volume). Lactate production was calculated as the increase of media lactate at 24, 48, 72, and 96 hours by correcting for the amount of lactate removed by sampling and added with media replacement. To examine the relationship between adipocyte carbon flux and leptin secretion in adipocytes cultured with metformin or vanadium, the amount of carbon released as lactate per amount of carbon taken up as glucose over 96 hours was calculated as lactate production/glucose utilization and expressed as a percentage (28). Cumulative leptin production was calculated as the change of media leptin concentrations at 24, 28, 72, and 96 hours with correction for the amount of leptin removed during sampling. The area under the curve for leptin production between 0 and 96 hours was calculated by the trapezoidal method. The experimental results from each adipocyte suspension prepared from a single animal were analyzed in relation to a control well from the same suspension. Given the

individual variation in leptin responses between animals and/or suspensions, it was not appropriate to compare means from control and treated adipocytes from different animals and/or suspensions. Therefore, the means for all the controls in the metformin (n = 18) and vanadium (n = 14) experiments contain a larger sample size (n) of animals and/or suspensions than all except the 1.0 mM metformin dose, which was studied in every experiment. Thus, when the number of wells with a particular concentration of metformin or vanadium differed from the total number of control wells (e.g., 0.5 mM metformin; n = 9), each result was compared only to that obtained in a corresponding control well from the same suspension in a pair-wise comparison. To examine the relationships between the medium concentrations of metformin or vanadium, glucose uptake, lactate production, and glucose conversion to lactate, and leptin secretion, simple and multiple linear regression analyses were performed with a statistics software package (StatView for Macintosh, Abacus Concepts, Inc., Berkeley, CA).

Because metformin exhibited toxic effects on adipocyte metabolism at concentrations greater than 5.0 mM, results from cultures incubated with metformin at concentrations greater than 5.0 mM were not included in these analyses. The relationship between lactate production from glucose and leptin secretion was also examined within the control groups alone. Data are expressed as means + SEM.

Results

Effects of Metformin

The effects of metformin on glucose uptake, lactate production, and leptin secretion were examined. Metformin at a concentration of 0.1 mM did not affect glucose uptake compared with the corresponding control suspensions containing insulin (0.16 nM) alone. At 0.25 mM, glucose uptake was increased (%Δ = +9 ± 8%), but the effect was not statistically significant perhaps due to the smaller number of trials (n = 5) performed at this concentration. Metformin stimulated glucose uptake at concentrations of 0.5 mM (+37 ± 10%, $p < 0.005$) and 1.0 mM (+62 ± 8%, $p < 0.0001$) compared with that in the corresponding control suspensions (Table 1, Figure 1). At 5.0 mM, glucose uptake was not significantly different from control. Higher concentrations of metformin (≥25.0 mM) markedly inhibited glucose uptake most likely reflecting a toxic effect of metformin at these very high concentrations. Metformin had no significant effect on lactate production at concentrations lower than 0.5 mM but increased lactate production at concentrations of 0.5 mM (+105 ± 43% , $p < 0.025$), 1.0 mM (+186 ± 31%, $p < 0.0001$), and 5.0 mM (+202 ± 52%, $p < 0.0025$) vs. insulin alone (Table 1, Figure 1). At concentrations of 25.0 mM, lactate production was markedly inhibited ($p < 0.0001$), because glucose utilization was almost completely suppressed. Concentrations of metformin of 0.5 mM and below did not affect the proportional conversion of glucose to lactate (Table 1). However, glucose conversion to lactate was increased at a concentration of 1.0 mM, and this effect was marked at 5.0 mM with more than twice the amount of glucose released as lactate (Table 1). Although 1.0 mM metformin did increase mean glucose uptake over control rates, a significantly larger proportion of the glucose that was taken up was released as lactate. The concentration of 0.5 mM was the only level of metformin that induced a significant increase of glucose utilization without increasing the proportion of glucose carbon released as lactate (Table 1).

At concentrations of metformin lower than 0.5 mM, leptin secretion was unaffected. With metformin at 0.5 mM, the area under the curve (AUC) for leptin secretion over 96 hours was significantly greater (+20.5 ± 9%, $p < 0.05$) than control (Figure 2). Metformin inhibited leptin secretion at concentrations of 1.0 mM (−25 ± 8%, $p < 0.005$), 5.0 mM (−89 ± 4%, $p < 0.0001$), and by 90% at toxic concentrations of 25.0 mM ($p < 0.0001$) (Figure 2).

Within the 18 control wells, leptin secretion was inversely related to the conversion of glucose to lactate ($r = -0.61$; $p < 0.01$). At metformin concentrations from 0 to 5.0 mM, leptin secretion was inversely proportional to the log of the metformin concentration ($r = -0.53$; $p < 0.0001$), to lactate production ($r = -0.53$; $p < 0.0001$), and to the proportional conversion of glucose to lactate across metformin doses ($r = -0.74$; $p < 0.0001$)(Figure 3) but was not related to glucose uptake ($r = 0.13$; $p = 0.27$) by simple regression. By multiple regression analysis, leptin secretion was inversely related to the conversion of glucose to lactate ($p < 0.0001$) but not to the log of the metformin concentration ($p = 0.91$), lactate production ($p = 0.39$), or glucose uptake ($p = 0.62$). Leptin secretion was only increased over control by metformin at 0.5 mM, which was also the only concentration that significantly increased glucose uptake without shunting a greater proportion of the glucose into lactate production (Table 1). A similar inverse relationship ($r = -0.73$; $p < 0.0025$) between leptin production and anaerobic glucose metabolism to lactate was observed in 32 control wells containing either no insulin or a low insulin concentration of 0.16 nM (~20 μU/mL) (Figure 4).

Effects of Vanadium

The effects of vanadium on glucose uptake, lactate production, and leptin secretion were examined in adipocytes cultured with concentrations of vanadium of 0 to 50 μM. Vanadium at 5.0 μM (+20 ± 7%, $p < 0.01$), 10.0 μM (+38 ± 12%, $p < 0.02$), and 20.0 μM (+34 ± 13%, $p < 0.02$) increased glucose uptake, compared with rates of glucose uptake in the corresponding control suspensions (Table 2, Figure 5). The effect of vanadium at these con-

Table 1. Effects of metformin in the presence of 0.16 nM insulin on glucose uptake, lactate production, and the percentage of glucose carbon taken up that was released as lactate by isolated rat adipocytes over 96 hours in culture (mean ± SEM)

[Metformin] (mM) + Insulin (0.16 nM)	Glucose uptake (μmol) over 96 hours	Lactate production (μmol) over 96 hours	Glucose to lactate (%)
Control (n = 18)	7.5 ± 0.7	5.7 ± 0.5	40.9 ± 3.6
0.1 (n = 4)	6.5 ± 0.9	5.9 ± 0.6	47.2 ± 6.3
0.25 (n = 5)	11.0 ± 1.4	8.6 ± 1.3	40.2 ± 5.9
0.5 (n = 9)	11.0 ± 1.2†	9.5 ± 1.2†	44.9 ± 5.1
1.0 (n = 18)	11.6 ± 0.7‡	14.4 ± 0.9‡	63.8 ± 3.4*
5.0 (n = 15)	8.3 ± 0.6	14.4 ± 1.0‡	85.6 ± 4.1‡

* p = 0.01; † p = 0.005; ‡ p = 0.0005; vs. corresponding controls from the same adipocyte suspensions.

centrations was comparable to that of insulin at 1.6 nM, which increased glucose uptake by 38 ± 8% (p < 0.0001)(Table 2, Figure 5). Vanadium at 50.0 μM did not significantly affect glucose uptake (%Δ = −4 ± 14%). Vanadium at 5.0 μM increased lactate production by + 17 ± 8% (p < 0.025). Mean lactate production in the six wells that served as controls for the 10.0 μM concentration of vanadium was lower than average; however, lactate production was increased in five of six corresponding experimental wells. Thus, vanadium at 10 μM increased lactate production by 61 ± 20% (p < 0.02) despite absolute lactate production being similar to the mean of the total 14 control wells. At 20.0 and 50.0 μM, lactate production was not significantly different from that of the control (Table 2).

Insulin at 1.6 nM increased leptin secretion over 96 hours by 59 ± 15% (p < 0.005) and the 0- to 96-hour AUC by 38 ± 8% (p < 0.0001)(Figure 5). Leptin secretion was unaffected by vanadium at a concentration of 5 μM. Higher concentrations of 10, 20, and 50 μM consistently inhibited leptin secretion over 96 hours by −33 ± 6% (p < 0.0025), −53 ± 7% (p < 0.0001), and −61 ± 8% (p < 0.001), respectively (Figure 6). Across vanadium concentrations, leptin secretion at 96 hours was positively correlated with glucose uptake (r = 0.35; p < 0.02) and inversely related to the log of the vanadium concentration (r = −0.44; p < 0.0001), to lactate production (r = −0.30; p < 0.025), and to the conversion of glucose to lactate (r = −0.58; p <

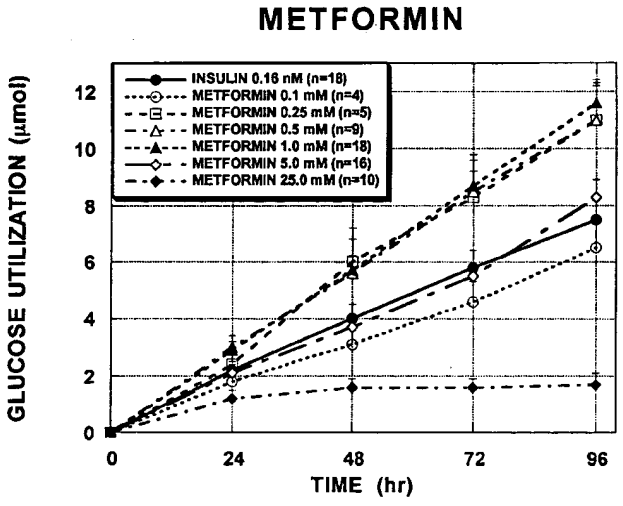

Figure 1. Glucose utilization (corrected for media sampling and replacement) over 96 hours by isolated rat adipocytes in primary culture with insulin at 0.16 nM and metformin at concentrations from 0 to 25.0 mM.

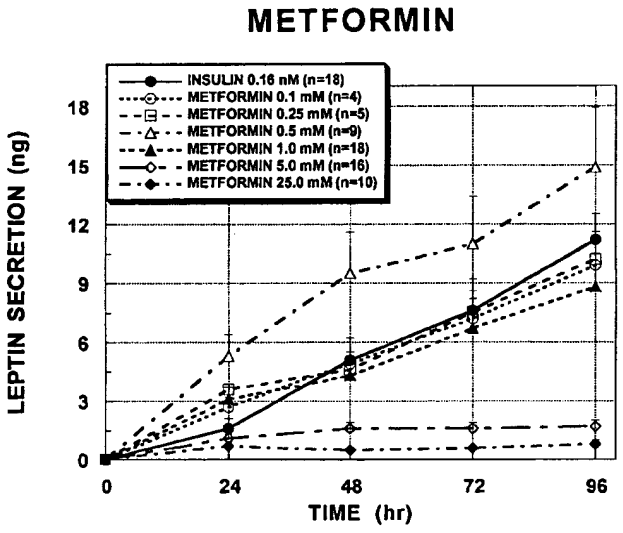

Figure 2. Leptin secretion (corrected for media sampling and replacement) over 96 hours by isolated rat adipocytes in primary culture with insulin at 0.16 nM and metformin at concentrations from 0 to 25.0 mM.

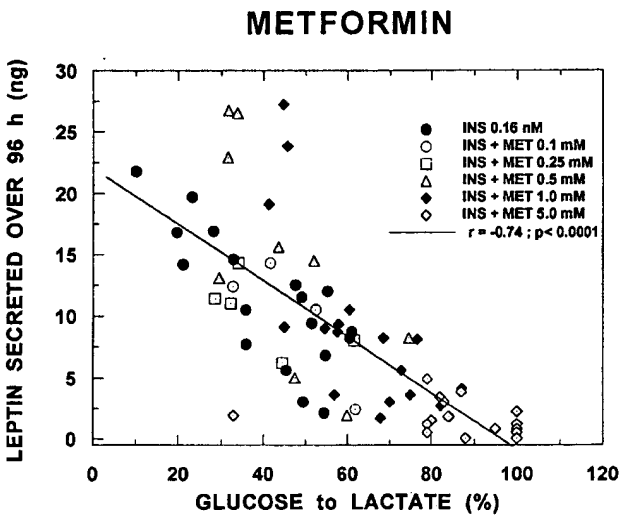

Figure 3. Relationship between the percentage of glucose taken up and released as lactate and leptin secretion over 96 hours by adipocytes in primary culture with insulin (INS) at 0.16 nM and metformin (MET) at concentrations from 0 to 5.0 mM. Leptin secretion, glucose utilization, and lactate production are corrected for media sampling and replacement.

0.0001) (data not shown). By multiple regression analysis, leptin secretion at 96 hours was more closely related to glucose conversion to lactate ($p < 0.0001$) than to absolute lactate production ($p < 0.02$) or the log of the vanadium concentration ($p < 0.005$) and was not related to absolute

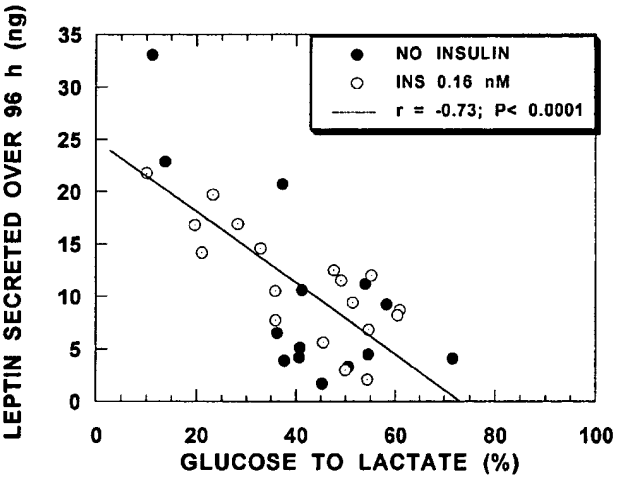

Figure 4. Relationship between the percentage of glucose taken up and released as lactate and leptin secretion over 96 hours by adipocytes in primary culture in 32 control wells containing no added insulin or insulin at a low concentration of 0.16 nM. Leptin secretion, glucose utilization, and lactate production are corrected for media sampling and replacement.

glucose uptake ($p = 0.22$). Despite the relationship between leptin secretion and the conversion of glucose to lactate across vanadium concentrations, unlike with metformin, the percentage of glucose released as lactate was not altered by any concentration of vanadium (Table 2). In contrast, insulin at 1.6 nM, which significantly decreased the proportional conversion of glucose to lactate (i.e., anaerobic glucose metabolism) (Table 2), stimulated leptin secretion (Figure 6).

Discussion

We have recently reported that insulin-mediated glucose metabolism is an important factor regulating leptin expression and secretion in isolated rat adipocytes (28). Some previous studies have shown that drugs in the thiazolidinedione class, which are used in the treatment of type 2 diabetes, can inhibit leptin production in vitro and in vivo (38,39). In the present study, we examined the effects of metformin and vanadium, two other antidiabetic drugs known to increase cellular glucose utilization, on leptin secretion, glucose uptake, and lactate production in isolated cultured rat adipocytes. Our goal was to use these compounds as tools to examine their effects for altering adipocyte glucose utilization on leptin secretion. Therefore, we used concentrations within a range that were found to produce significant increases of adipocyte glucose uptake. Particularly in the case of metformin, these concentrations (0.25 to 5.0 mM) were far above the range of plasma concentrations (0.005 to 0.02 mM) observed in patients treated with therapeutic doses of metformin (40). In fact, at therapeutic concentrations, metformin seems to act primarily to inhibit hepatic glucose production with limited, if any, effects on peripheral glucose uptake (41–43). At therapeutic concentrations, metformin generally has little direct effect on glucose utilization in vitro (44,45). At concentrations higher than those achieved in serum with therapeutic metformin administration, metformin stimulates glucose transport by rat (46,47) and human adipocytes (48), and in rat and human skeletal muscle (see reviews in 29,34,48,49). At the cellular level, high concentrations of metformin increase insulin receptor binding, along with tyrosine kinase activity, glucose transport, and glycogen synthesis (33,34).

In the present study, metformin concentrations ranging from 0.5 to 5.0 mM increased both glucose uptake and lactate production. In addition to increasing absolute lactate production, metformin at 1.0 and 5.0 mM increased the percentage of glucose carbon that was metabolized to lactate and released into the culture media by 80% to 170%. At high concentrations of metformin (= 25.0 mM), both glucose uptake and lactate production were markedly inhibited, most likely due to a toxic effect of very high levels of metformin on cellular metabolism. Metformin at 0.5 mM modestly increased leptin secretion by ~20%.

Table 2. Effects of insulin (1.6 nM) or vanadium on glucose uptake, lactate production, and the percentage of glucose carbon taken up that was released as lactate by isolated rat adipocytes over 96 hours in culture (mean ± SEM)

[Vanadium] (μM); no insulin added	Glucose uptake (μmol) over 96 hours	Lactate production (μmol) over 96 hours	Glucose to lactate (%)
Control (n = 14)	6.8 ± 0.5	5.6 ± 0.6	42.4 ± 4.3
1.6 nM Ins (n = 14)	9.4 ± 0.9§	5.7 ± 0.7	33.0 ± 3.5‡
5.0 (n = 12)	7.8 ± 1.1§	5.7 ± 0.6*	40.5 ± 4.7
10.0 (n = 6)	8.2 ± 1.3‡	5.2 ± 0.8†	36.0 ± 6.5
20.0 (n = 12)	8.7 ± 1.2‡	6.3 ± 0.6	41.6 ± 5.0
50.0 (n = 13)	6.9 ± 1.02	4.9 ± 0.5	53.3 ± 9.4

*p = 0.05; †p = 0.02; ‡p = 0.0025; §p = 0.0005; vs. corresponding control wells from the same adipocyte suspensions.

Importantly, this was the only concentration of metformin tested that increased glucose uptake without shunting a greater proportion of glucose into lactate production. In contrast, at concentrations of 1.0 mM and higher, leptin secretion was modestly to markedly suppressed.

A significant proportion of glucose taken up by adipose tissue is metabolized to lactate and released (50). At metformin concentrations ≤ 5.0 mM, leptin secretion was inversely related to the amount of glucose taken up by the adipocytes, converted to lactate, and released into the media. We have previously observed that the stimulation of leptin secretion by insulin is associated not only with increased glucose utilization, but with a decrease in the proportional conversion of glucose to lactate (28), a finding that was also observed within the control groups in the present study (Figure 4). Thus, when lactate production is increased, less carbon derived from glucose is available to enter the tricarboxylic acid cycle either for oxidation or use in de novo lipogenesis. Together, these data suggest that it is not glucose uptake, per se, but its metabolism beyond pyruvate and lactate in the adipocyte that is involved in the action of glucose to stimulate leptin secretion. Thus, the anaerobic metabolism of glucose does not stimulate leptin production. The entry of glucose into the hexosamine biosynthetic pathway and the production of UDP-glucosamine have been suggested as a mechanism by which glucose utilization can

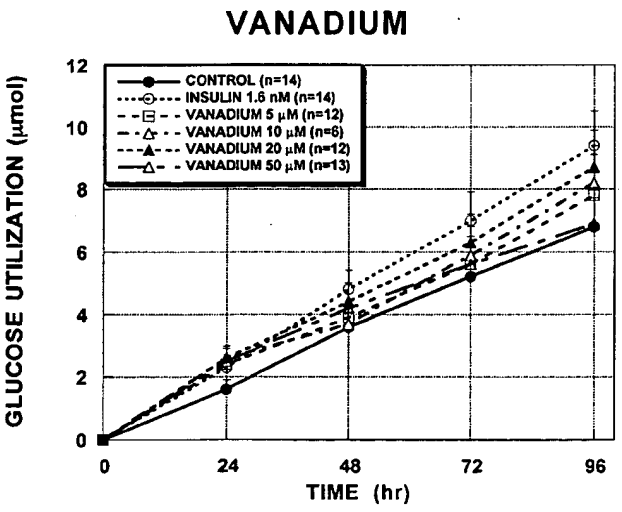

Figure 5. Glucose utilization (corrected for media sampling and replacement) over 96 hours in by isolated rat adipocytes in primary culture with vanadium at concentrations from 0 to 50.0 μM or with insulin at 1.6 nM.

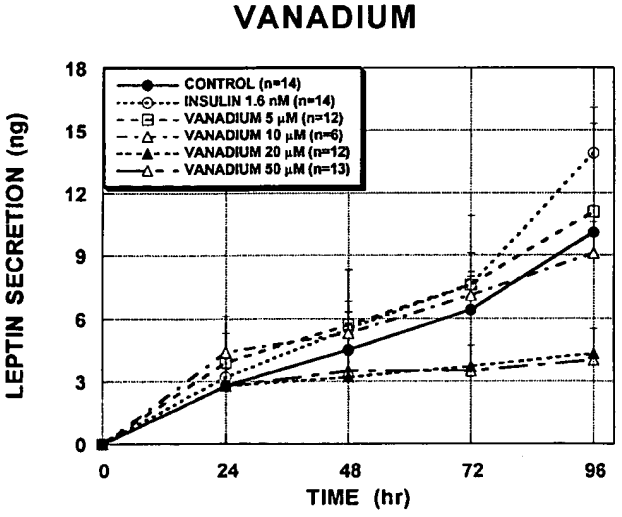

Figure 6. Leptin secretion (corrected for media sampling and replacement) over 96 hours by isolated rat adipocytes in primary culture with vanadium at concentrations from 0 to 50.0 μM or with insulin at 1.6 nM.

stimulate leptin production in adipose tissue (51). However, our results indicate that glucose can be metabolized to lactate, a point well beyond where glucose enters the hexosamine pathway, without stimulating leptin production. Therefore, if the hexosamine pathway were to be the sole mechanism by which glucose regulates leptin production, one would need to postulate that either anaerobic glucose metabolism and/or metformin itself, have an inhibitory effect on glucose entry into this pathway. The results of the present study suggest that it is more likely that the effect of glucose metabolism to stimulate leptin production involves glucose oxidation and/or the production of lipogenic precursors.

Metformin at 0.1 and 0.25 mM did not effect glucose metabolism or leptin secretion. Thus, metformin at therapeutic levels is unlikely to affect leptin production in vivo. Of the concentrations of metformin tested in this study, only 0.5 mM increased glucose uptake without shunting a larger proportion of the glucose into lactate. As previously discussed, this was the only concentration of metformin that had effects on glucose metabolism that did not inhibit leptin secretion, and, in fact, leptin secretion was modestly increased at 0.5 mM. Thus, only when glucose uptake *and* its metabolism beyond lactate were simultaneously increased, did we observe an increase, and not an inhibition, of leptin secretion. Therefore, it seems that only within a very narrow concentration range is metformin able to have a net effect to increase glucose uptake as well as its metabolism beyond lactate in isolated adipocytes. Accordingly, the effects of metformin to inhibit leptin secretion at most concentrations examined is likely to be a result of its effects to direct pyruvate metabolism into lactate and away from other potential pathways for pyruvate metabolism such as oxidation or lipogenesis.

The use of vanadium-containing compounds in the treatment of diabetes has been widely investigated in animals (52,53), and a few clinical trials have been conducted in human patients (54,55). A compound structurally similar to the form of organified vanadium used in this study (BMOV) has recently entered Phase 1 clinical trials. To our knowledge, the present study is the first report examining the effects of a vanadium compound on leptin production in vivo or in vitro.

Vanadium stimulated glucose uptake at concentrations up to 20 μM, whereas glucose uptake was not affected by a concentration of 50 μM. Lactate production was modestly increased at the lower concentrations of vanadium. We found that vanadium at a low concentration of 5.0 μM did not affect leptin production, however, concentrations of 10.0 μM and higher inhibited leptin secretion from isolated adipocytes by 30% to 60%. Although the amount of leptin secreted was inversely proportional to the percentage conversion of glucose to lactate across the concentrations of vanadium tested, this relationship was significantly weaker than that observed across metformin concentrations.

Furthermore, the proportion of glucose taken up and released as lactate was unaffected by vanadium at any concentration. Thus, in contrast to what was observed with metformin, the ability of vanadium to inhibit leptin secretion seems to be independent of any effects on glucose metabolism or lactate production, most likely because it does not increase the proportion of glucose fluxing into anaerobic metabolism.

The observed effects of vanadium result from one or more of the multiple known biological actions of vanadium in cells. These include the inhibition of protein tyrosine phosphatases and the activation of cytosolic protein-tyrosine kinases, resulting in the alteration of cellular tyrosine phosphorylation content (30,56). Vanadium has also been shown to exert direct inhibitory effects on a number of other cellular enzymes, including acid, alkaline, and dual-function phosphatases, ATPases, glucose-6-phosphatase, and fructose-2,6-bisphosphatase (30,55). At high concentrations, vanadium might exert some toxic effects on the cells, an effect which could underlie the lack of effect of the highest concentration of vanadium to stimulate glucose uptake, as well as the inhibition of leptin production at the two highest concentrations examined. In particular, the effects of vanadium to inhibit the activity of one or more enzymes involved in cellular energy metabolism could both inhibit leptin production and, at high concentrations, impair the ability of the cell to utilize energy derived from glucose metabolism.

In vanadium-treated animals, plasma vanadium concentrations have been estimated to be in the 10 to 20 μM range and in human clinical trials in the 1 to 5 μM range (53). Although it is unlikely based on the present results that the concentration of vanadium achieved in humans would be sufficient to affect leptin production, previous human studies employed low doses of vanadyl sulfate or sodium metavanadate, which are molecular forms that exhibit poor bioavailability. The potential effects on leptin secretion of the more readily absorbed forms of vanadium, such as the organified vanadium compound (BMOV) used in the present study (31), should therefore be considered.

In summary, both metformin and vanadium inhibit leptin secretion from primary cultures of rat adipocyte at concentrations that significantly increase glucose utilization. The inhibition of leptin production by metformin, but not by vanadium, is related to an increased conversion of glucose to lactate (i.e., anaerobic metabolism). This effect of metformin, coupled with our previous findings (28), suggests that the effect of glucose utilization to stimulate leptin production is not mediated by glucose uptake per se but involves the metabolism of glucose beyond pyruvate to a fate other than lactate, possibly oxidation or lipogenesis. Thus, metformin is a useful tool for examining the effects of

increasing anaerobic glucose metabolism. Further research, including examination of the potential roles of glucose oxidation and lipogenesis, needs to be conducted to determine the precise biochemical and molecular mechanisms by which glucose metabolism regulates leptin production.

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From genes to protein structure and function: novel applications of computational approaches in the genomic era

Jeffrey Skolnick and Jacquelyn S. Fetrow

The genome-sequencing projects are providing a detailed 'parts list' of life. A key to comprehending this list is understanding the function of each gene and each protein at various levels. Sequence-based methods for function prediction are inadequate because of the multifunctional nature of proteins. However, just knowing the structure of the protein is also insufficient for prediction of multiple functional sites. Structural descriptors for protein functional sites are crucial for unlocking the secrets in both the sequence and structural-genomics projects.

Genome-sequencing projects are providing a detailed 'parts list' for life. Unfortunately, this list, a portion of which represents the amino acid sequence of all the proteins in a given genome, does not come with an instruction manual. That is, given the genome's sequences, one does not necessarily know straight away which regions encode proteins, which serve a regulatory role and which are responsible for the structure and replication of the DNA itself.

This is not unlike giving a child a list of parts necessary to create a working automobile. Without the necessary expertise, creating the final, working car from just the initial parts list is a nearly impossible task. Similarly, understanding how to create a complete, functioning cell given just the sequence of nucleotides found in an organism's genome is a complex problem.

What is a protein function?

After a genome is sequenced and its complete parts list determined, the next goal is to understand the function(s) of each part, including that of the proteins. What do we mean by protein function, the focus of this article?

Function has many meanings. At one level, the protein could be a globular protein, such as an enzyme, hormone or antibody, or it could be a structural or membrane-bound protein. Another level is its biochemical function, such as the chemical reaction and the substrate specificity of an enzyme. The regulatory molecules or cofactors that bind to a protein are also levels of biochemical function.

At the cellular level, the protein's function would involve its interaction with other macromolecules and the function and cellular location of such complexes. There is also the protein's physiological function; that is, in which metabolic pathway the protein is involved or what physiological role it performs in the organism. Finally, the phenotypic function is the role played by the protein in the total organism, which is observed by deleting or mutating the gene encoding the protein.

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Obviously, the complete characterization of protein function is difficult but efforts are under way at all levels[1–4], including cellular function[5,6]. In this article, however, we focus on identifying the biochemical function of a protein given its sequence, a problem that is amenable to molecular approaches.

Sequence-based approaches to function prediction

The sequence-to-function approach is the most commonly used function-prediction method. This robust field is well developed and, in the interest of space limitations, we will merely present a brief overview.

There are two main flavors of this approach: sequence alignment[7–9]; and sequence–motif methods such as Prosite[10], Blocks[11], Prints[12,13] and Emotif[14]. Both the alignment and the motif methods are powerful but a recent analysis has demonstrated their significant limitations[15], suggesting that these methods will increasingly fail as the protein-sequence databases become more diverse.

An extension of these approaches that combines protein-sequence with structural information has been developed and some successes have been reported[16]. However, this method still applies the structural information in a one-dimensional, 'sequence-like' fashion and fails to take into account the powerful three-dimensional information displayed by protein structures.

In addition, proteins can gain and lose function during evolution and may, indeed, have multiple functions in the cell (Box 1). Sequence-to-function methods cannot specifically identify these complexities. Inaccurate use of sequence-to-function methods has led to significant function-annotation errors in the sequence databases[17].

An alternative approach

An alternative, complementary approach to protein-function prediction uses the sequence-to-structure-to-function paradigm. Here, the goal is to determine the structure of the protein of interest and then to identify the functionally important residues in that structure. Using the chemical structure itself to identify functional sites is more in line with how the protein actually works.

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In a sense, this is one long-term goal of 'structural genomics' projects[18,19], which are designed to determine all possible protein folds experimentally, just as genome-sequencing projects are determining all protein sequences[20]. This is in contrast to traditional structural-biology approaches, in which one knows the protein's function first and only then, if the function is sufficiently important, determines its structure.

It is implicitly assumed that having the protein's structure will provide insights into its function, thereby furthering the goals of the human-genome-sequencing project. However, knowing a protein's three-dimensional structure is insufficient to determine its function (Box 2). What we really need to analyse and predict the multifunctional aspects of proteins is a method specifically to recognize active sites and binding regions in these protein structures.

Active-site identification

In order to use a structure-based approach to function prediction, one must identify the key residues responsible for a given biochemical activity. For many years, it has been suggested that the active sites in proteins are better conserved than the overall fold. Taken to the limit, this suggests that one could not only identify distant ancestors with the same global fold and the same activity but also proteins with similar functions but distantly related, or possibly unrelated, global folds.

The validity of this suggestion was demonstrated empirically by Nussinov and co-workers, who showed that the active sites of eukaryotic serine proteases, subtilisins and sulfhydryl proteases exhibit similar structural motifs[21]. Furthermore, in a recent modeling study of *Saccharomyces cerevisiae* proteins, protein functional sites were found to be more conserved than other parts of the protein models[22]. Similarly, it has been demonstrated that the catalytic triad of the α/β hydrolases is structurally better conserved than other histidine-containing triads[23]. A comparison of the structure of the hydrolase catalytic triad to other histidine-containing triads shows a distinct bimodal distribution, while a similar analysis done with a randomly selected triad shows a unimodal distribution (Fig. 1).

Kasuya and Thornton[24] generalized this example by creating structural analogs of a few Prosite sequence motifs[10]. For the 20 most-frequently occurring Prosite patterns, the associated local structure is quite distinct. These results provide clear evidence that enzyme active sites are indeed more highly conserved than other parts of the protein.

Identifying active sites in experimental structures

Historically, several groups have attempted to identify functional sites in proteins; these efforts were directed at protein engineering or building functional sites in places where they did not previously exist. This has been successfully accomplished for several metal-binding sites[25–33]. However, highly accurate functional-site descriptors of the backbone and side-chain atoms were required, fueling the belief that significant atomic detail is required in site descriptors for function identification.

Highly detailed residue side-chain descriptors of the active sites of serine proteases and related proteins have been used to identify functional sites[3]. The use of these highly detailed motifs has led to the identification of several novel functional sites in known, high-quality protein structures[3,34]. More automated methods for finding spatial motifs in protein structures have also been described[21,34–40].

Unfortunately, most of these methods require the exact placement of atoms within protein backbones and side chains, and so have not been shown to be relevant to inexact predicted structures. Recently, however, we described the production of fuzzy, inexact descriptors of protein functional sites[15]. As we wish to apply the descriptors to experimental structures as well as to predicted protein models, we used only carbon atoms and side-chain centers-of-mass positions. We call these descriptors 'fuzzy functional forms' (FFFs) and have created them for both the disulfide-oxidoreductase[15,41] and α/β-hydrolase catalytic active sites[23].

The disulfide-oxidoreductase FFF was applied to screen high-resolution structures from the Brookhaven protein database[42]. In a dataset of 364 protein structures, the FFF accurately identified all proteins known to exhibit the disulfide-oxidoreductase active site[15]. In a larger dataset of 1501 proteins, the FFF again accurately identified all proteins with the active site. In addition, it identified another protein, 1fjm, a serine–threonine phosphatase. This result was initially discouraging but subsequent sequence alignment and clustering analysis strongly suggested that this putative site might indeed be a site of redox regulation in the serine–threonine phosphatase-1 subfamily[43]. If confirmed by experiment, this result will highlight the advantages of using structural descriptors to analyse multiple functional sites in proteins. It will also highlight the fact that human

Box 1. Proteins are multifunctional

A common protein characteristic that makes functional analysis based only on homology especially difficult is the tendency of proteins to be multifunctional. For instance, lactate dehydrogenase binds NAD, substrate and zinc, and performs a redox reaction. Each of these occurs at different functional sites that are in close proximity and the combination of all four sites creates the fully functional protein.

Other examples of multifunctional proteins are the nucleic-acid-binding proteins. For instance, DNA regulatory proteins often contain a DNA-binding domain, a multimerization domain and additional sites that bind regulatory proteins; a classic example is RecA[59]. The 3C rhinovirus protease exhibits a proteolytic function as well as an RNA-binding function[60,61]. Transcription factors are also complex, multifunctional proteins[62]. It is becoming increasingly important to recognize each of these different functions of gene products of a newly sequenced gene.

The serine–threonine-phosphatase superfamily is a prime example of the difficulties of using standard sequence analysis to recognize the multiple functions found in single proteins. This large protein family is divided into a number of subfamilies, all of which contain an essential phosphatase active site. Subfamilies 1, 2A and 2B exhibit 40% or more sequence identity between them[63]. However, each of these subfamilies is apparently regulated differently in the cell[64–67] and observation suggests that there are different functional sites at which regulation can occur. Because the sequence identity between subfamilies is so high, standard sequence-similarity methods could easily misclassify new sequences as members of the wrong subfamily if the functional sites are not carefully considered, as was recently demonstrated[43].

These are but a few examples of the multifunctionality of proteins. The recognition of this multifunctional nature is of critical importance to the genomics field. Useful functional-annotation methods must consider all of the specific functions in a given protein and will not just provide a general classification of function.

Box 2. Knowing a protein's structure does not necessarily tell you its function

Because proteins can have similar folds but different functions[68,69], determining the structure of a protein may or may not tell you something about its function. The most well-studied example is the $(\alpha/\beta)_8$ barrel enzymes, of which triose-phosphate isomerase (TIM) is the archetypal representative. Members of this family have similar overall structures but different functions, including different active sites, substrate specificities and cofactor requirements[70,71].

Is this example common? Our own analysis of the 1997 SCOP database[68] shows that the five largest fold families are the ferredoxin-like, the (α/β) barrels, the knottins, the immunoglobulin-like and the flavodoxin-like fold families with 22, 18, 13, 9 and 9 subfamilies, respectively (Fig. i). In fact, 57 of the SCOP fold families consist of multiple superfamilies. These data only show the tip of the iceberg, because each superfamily is further composed of protein families and each individual family can have radically different functions. For example, the ferredoxin-like superfamily contains families identified as Fe–S ferredoxins, ribosomal proteins, DNA-binding proteins and phosphatases, among others.

After this article was submitted, a much-more-detailed analysis of the SCOP database was published[72]. This finds a broad function–structure correlation for some structural classes, but also finds a number of ubiquitous functions and structures that occur across a number of families. The article provides a useful analysis of the confidence with which structure and function can be correlated[72]. Knowing the protein structure by itself is insufficient to annotate a number of functional classes and is also insufficient for annotating the specific details of protein function.

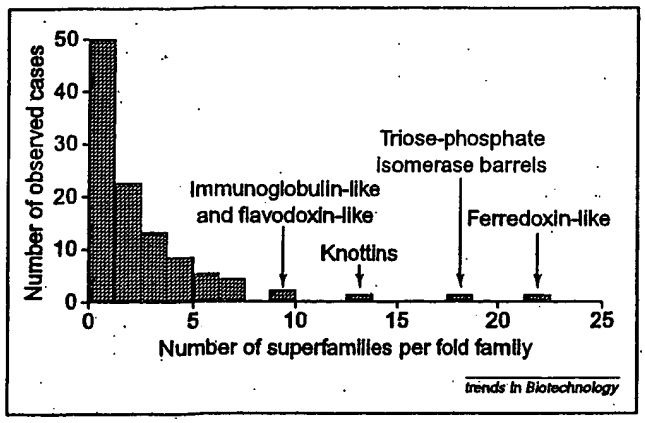

Figure i

Histogram of the numbers of superfamilies found in each SCOP fold family. These data clearly show that proteins with similar structures can have different functions and demonstrate the difficulty of assigning protein function based simply on the three-dimensional structure. The data were taken from the 1997 distribution of SCOP (http://scop.mrc-lmb.cam.ac.uk/scop). For a more-detailed analysis, see Ref. 72.

observation alone is no longer adequate for identifying all functional sites in known protein structures.

To date, the use of structure to identify function has largely focused on high-resolution structures and highly detailed descriptors of protein functional sites. However, the creation of inexact descriptors for functional sites opens the way to the application of these methods to inexact, predicted protein models. The question remains: how good does a model have to be in order to use FFFs to identify its active sites?

The state of the art in structure-prediction methods

For proteins whose sequence identity is above ~30%, one can use homology modeling to build the structure[44]. However, structure prediction is far more difficult for proteins that are not homologous to proteins with known structure. At present, there are two approaches for these sequences: *ab initio* folding[45–48] and threading[49–53].

In *ab initio* folding, one starts from a random conformation and then attempts to assemble the native structure. As this method does not rely on a library of pre-existing folds, it can be used to predict novel folds. The recent CASP3 protein-structure-prediction experiment (http://PredictionCenter.llnl.gov/CASP3) involved the blind prediction of the structure of proteins whose actual structure was about to be experimentally determined. These results indicate that considerable progress has been made[46,54]. For helical and α/β proteins with less than 110 residues, structures were often predicted whose backbone root-mean-square deviation (RMSD) from native ranged from 4–7 Å. Progress is being made with the β proteins, too, although they remain problematic. Because *ab initio* methods can identify novel folds, these methods could be used to help to select sequences likely to yield novel folds in experimental structural-genomics projects.

Another approach to tertiary-structure prediction is threading. Here, for the sequence of interest, one attempts to find the closest matching structure in a library of known folds[52,55]. Threading is applicable to proteins of up to 500 residues or so and is much faster than *ab initio* approaches. However, threading cannot be used to obtain novel folds.

Ab initio *predicted models can be used for automatic protein-function prediction*

The results of the recent CASP3 competition suggest that current modeling methods can often (but not always) create inexact protein models. Are these structures useful for identifying functional sites in proteins? Using the *ab initio* structure-prediction program MONSSTER, the tertiary structure of a glutaredoxin, 1ego, was predicted[56]. For the lowest-energy model, the overall backbone RMSD from the crystal structure was 5.7 Å.

To determine whether this inexact model could be used for function identification, the sets of correctly and incorrectly folded structures were screened with the FFF for disulfide-oxidoreductase activity[15]. The FFF uniquely identified the active site in the correctly folded structure but not in the incorrectly folded ones (Fig. 2). This is a proof-of-principle demonstration that inexact models produced by *ab initio* prediction of structure from sequence can be used for the subsequent prediction of biochemical function. Of course, improvements in the method have to be made before such predictions can be done on a routine basis.

Use of predicted structures from threading in protein-function prediction

At present, practical limitations preclude folding an entire genome of proteins using *ab initio* methods[57]. Threading is more appropriate for achieving the requisite high-throughput structure prediction. Thus, a standard threading algorithm[58] has been used to screen all

proteins in nine genomes for the disulfide-oxidoreductase active site described above.

First, sequences that aligned with the structures of known disulfide oxidoreductases were identified. Then, the structure was searched for matches to the active-site residues and geometry. For those sequences for which other homologs were available, a sequence-conservation profile was constructed[23]. If the putative active-site residues were not conserved in the sequence subfamily to which the protein belongs, that sequence was eliminated. Otherwise, the sequence is predicted to have the function.

Using this sequence-to-structure-to-function method, 99% of the proteins in the nine genomes that have known disulfide-oxidoreductase activity have been found. From 10% to 30% more functional predictions are made than by alternative sequence-based approaches; similar results are seen for the α/β hydrolases[23]. Surprisingly, in spite of the fact that threading algorithms have problems generating good sequence-to-structure alignments, active sites are often accurately aligned, even for very distant matches. This observation would agree with the above experimental results indicating that active sites are well conserved in protein structures.

Importantly, the false-positive rate when using structural information is much lower than that found using sequence-based approaches, as demonstrated by a detailed comparison of the FFF structural approach and the Blocks sequence-motif approach (N. Siew *et al.*, unpublished). In this study, the sequences in eight genomes, including *Bacillus subtilis*, were analysed for disulfide-oxidoreductase function using the disulfide-oxidoreductase FFF, the thioredoxin Block 00194 and the glutaredoxin Block 00195. If we assume that those sequences identified by both the FFF and Blocks are 'true positives', we find 13 such sequences in the *B. subtilis* genome.

There is no experimental evidence validating all of these 'true positives' and so they are more accurately termed 'consensus positives'. In order to find these 13 'consensus positive' sequences, the FFF hits seven false positives. On the other hand, Blocks hits 23 false positives (Fig. 3). It was previously suggested that the use of a functional requirement adds information to threading and reduces the number of false positives[52]. These data, including the data shown in Fig. 3, validate this claim on a genome-wide basis.

Of course, as no genome has had the function of all of its proteins experimentally annotated, it is impossible to know how many other proteins with the specified biochemical function were not properly identified. This is a critical question for researchers attempting to predict protein function. Experimental confirmation will be needed to validate this or any other method fully. This points out the need for closely coupling computational function-prediction algorithms with experiments.

Weaknesses of using the sequence-to-structure-to-function method of function prediction

Based on studies to date, the identification of enzymatic activity requires a model in which the backbone RMSD from native near the active sites is about 4–5 Å. Predicted models are better at describing the geometry in the core of the molecule than in the loops and so predicting the function of a protein whose active site is in loops may be a problem. Also, the method can currently only be applied to enzyme active sites; substrate- and ligand-binding sites have not been identified using the inexact models. Techniques that will further refine inexact protein models will be quite useful in taking the protein analysis to the next step.

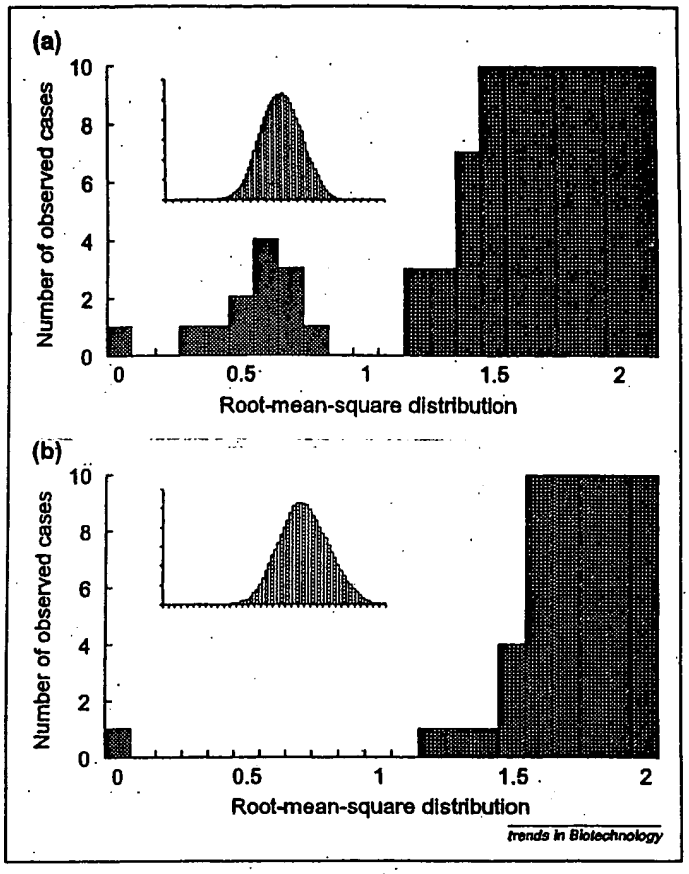

Figure 1

The distribution of root-mean-square distributions (RMSD) between the hydrolase catalytic triad and all other histidine-containing triads shows a bimodal distribution (a); by contrast, the RMSD between a randomly selected (non-catalytic) triad and all other histidine-containing triads has a unimodal distribution (b). The His–Ser–Asp catalytic trial in the protein-1 gpl (Rp2 lipase) (a) and a random histidine-containing triad from 4pga (glutaminase–asparaginase) (b) were structurally aligned to all His-containing triads in a database of 1037 proteins[23]. Actual α/β-hydrolase active sites (a) and the 4pga site (b) are indicated by blue bars; other histidine triads that are not active sites are indicated by red bars. None of the sites found by matching to the 4pga were hydrolase active sites. Inset graphs show the full distribution.

Conclusions

Although sequence-based approaches to protein-function prediction have proved to be very useful, alternatives are needed to assign the biochemical function of the 30–50% of proteins whose function cannot be assigned by any current methods. One emerging approach involves the sequence-to-structure-to-function paradigm. Such structures might be provided by structural-genomics projects or by structure-prediction algorithms. Functional assignment is made by screening the resulting structure against a library of structural descriptors for known active sites or binding regions.

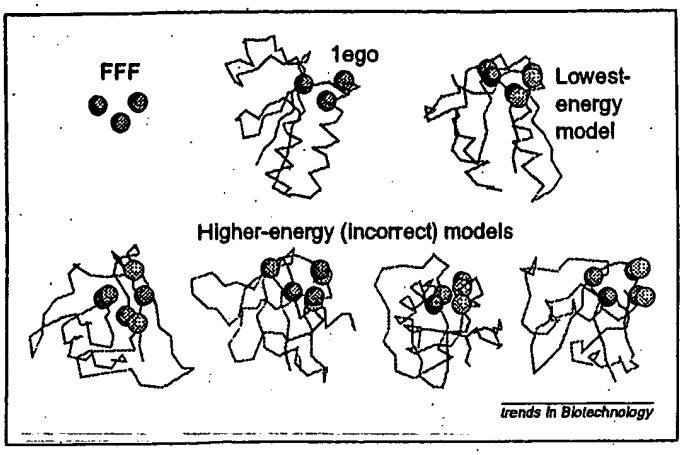

Figure 2

Application of the disulfide-oxidoreductase fuzzy functional form (FFF) to *ab initio* models of glutaredoxin created by the program MONSSTER shows that the FFF can distinguish between correctly folded and misfolded (or higher-energy) models. The FFF is shown as two orange balls (representing the cysteines) and a blue ball (representing the proline). The protein models are shown as magenta wire models with the active-site cysteines and proline shown as yellow and cyan balls, respectively. The FFF clearly distinguishes the correct active site in the crystal structure of the glutaredoxin 1ego and the correctly folded, lowest-energy model. The FFF does not match to the active sites of any of the higher energy, misfolded structures, four of which are shown here.

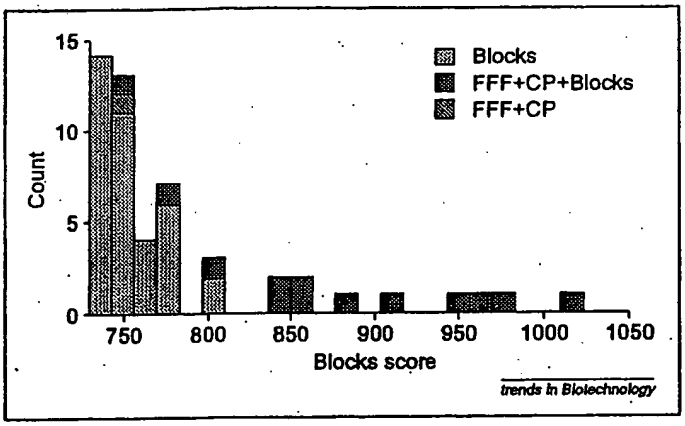

Figure 3

Analysis of the *Bacillus subtilis* genome using the thioredoxin Block 00194. The Blocks score (computed using the publicly available BLIMPS program) is plotted on the *x* axis and the number of sequences found in each scoring bin is plotted on the *y* axis. Those sequences identified as 'consensus positives' (identified by both the fuzzy functional form (FFF) and the Block) are shown as red bars. One additional sequence found by the FFF, which is likely to be a true positive, is shown as a blue bar. All other sequences, putative 'false positives', are shown as yellow bars. Using the Blocks score at which all 13 of the 'consensus positives' are found, 23 false positives are also found. In its analysis of the *B. subtilis* genome, the FFF identifies only seven false positives along with the same 13 'consensus positives' (data not shown).

Detailed descriptors will only work on the experimentally determined, high-quality structures. Ideally, however, the descriptors should work on both experimental structures and the cruder models provided by tertiary-structure-prediction algorithms.

The advantages of such an approach are that one need not establish an evolutionary relationship in order to assign function, that more than one function can be assigned to a given protein [an issue of major importance, because proteins are multifunctional (Box 1)] and, ultimately, that having a structure can provide deeper insight into the biological mechanism of protein function and regulation. The disadvantages are that one needs to have the protein's structure before a function can be assigned and that the approach is limited to those functions associated with proteins with at least one solved structure, so that a functional-site descriptor can be constructed.

In this sense, structure-to-function assignment can be thought of as 'functional threading' – find the active-site match in a library of descriptors for known protein active sites. This is the first step in the long process of using structure to assign all levels of function, a goal that is made increasingly important with the emergence of structural genomics. Based on the progress to date, it is apparent that structure will play an important role in the post-genomic era of biology.

Acknowledgment

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Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis

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Summary

Oligonucleotide-directed mutagenesis of *ctxB* was used to produce mutants of cholera toxin B subunit (CT-B) altered at residues Cys-9, Gly-33, Lys-34, Arg-35, Cys-86 and Trp-88. Mutants were identified phenotypically by radial passive immune haemolysis assays and genotypically by colony hybridization with specific oligonucleotide probes. Mutant CT-B polypeptides were characterized for immunoreactivity, binding to ganglioside GM1, ability to associate with the A subunit, ability to form holotoxin, and biological activity. Amino acid substitutions that caused decreased binding of mutant CT-B to ganglioside GM1 and abolished toxicity included negatively charged or large hydrophobic residues for Gly-33 and negatively or positively charged residues for Trp-88. Substitution of lysine or arginine for Gly-33 did not affect immunoreactivity or GM1-binding activity of CT-B but abolished or reduced toxicity of the mutant holotoxins, respectively. Substitutions of Glu or Asp for Arg-35 interfered with formation of holotoxin, but none of the observed substitutions for Lys-34 or Arg-35 affected binding of CT-B to GM1. The Cys-9, Cys-86 and Trp-88 residues were important for establishing or maintaining the native conformation of CT-B or protecting the CT-B polypeptide from rapid degradation *in vivo*.

Introduction

The symptoms of cholera, an acute diarrhoeal disease of humans caused by *Vibrio cholerae*, are due mainly to the effects of cholera toxin (CT) on the small intestine. CT and the heat-labile enterotoxins (LT) of *Escherichia coli* are structurally and functionally related and constitute the *V. cholerae/E. coli* enterotoxin family (reviewed by Finkelstein *et al.*, 1987). Type I and type II enterotoxins belong to distinct serogroups, and minor antigenic variants exist within each serogroup (Holmes *et al.*, 1986; Guth *et al.*, 1986; Pickett *et al.*, 1987; 1989). The sequences of the structural genes for representative type I and type II enterotoxins have been determined (reviewed by Betley *et al.*, 1986; Pickett *et al.*, 1987; 1989). CT and LT are multisubunit toxins of the A–B type (Olsnes *et al.*, 1990). CT is an 85 kDa complex containing a 27 kDa A polypeptide and five 11.6 kDa B polypeptides. The mature CT-B monomer consists of 103 amino acid residues (Lai, 1977), with a single intrachain disulphide link between Cys-9 and Cys-86. There are five ganglioside GM1 (GM1)-binding sites per molecule of CT (Fishman *et al.*, 1978). Interaction of the B pentamer of CT with GM1 on the plasma membrane of target cells initiates delivery of the A polypeptide to the cytosol. The A subunit is proteolytically cleaved to produce two polypeptides (A1 and A2) of 21.8 and 5.4 kDa, linked by a disulphide bond. The A1 polypeptide is the enzymatically active component of the toxin, catalysing the transfer of the ADP-ribose moiety from NAD to the α subunit of the Gs regulatory protein of adenylate cyclase. This activates adenylate cyclase, increases production of cAMP, and causes secretion of fluid and electrolytes by the small intestine (Field *et al.*, 1989).

Attempts to characterize the ganglioside-binding sites of CT and LT have concentrated on analysis of chemically modified toxins and on the isolation of mutants. However, the precise nature of the ganglioside-binding sites on CT-B and LT-B remains to be elucidated.

Several lines of evidence show that Trp-88 is closely associated with the GM1-binding site. Binding of GM1 to CT causes a blue shift of approximately 12 nm in the fluorescence emission maximum of the single tryptophan residue (Mullin *et al.*, 1976), and modification of tryptophan with nitrophenyl derivatives (de Wolf *et al.*, 1981) or formic acid (Ludwig *et al.*, 1985) destroys the ability of CT to bind to GM1. One or more of the nine lysine residues in CT-B appear(s) to be involved in the GM1 binding site (Ludwig *et al.*, 1985). Modification of CT-B with cyclohexanedione led Duffy and Lai (1979) to conclude that Arg-35 is involved in receptor binding, but Ludwig *et al.* (1985) could not attribute the effects of arginine modification to a specific residue from the three arginines in CT-B. The disulphide bond between Cys-9 and Cys-86 is important for the structural integrity of CT-B, and modification of

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these cysteines destroys both GM1-binding activity and antigenicity of CT-B (Ludwig *et al.*, 1985).

Several factors can complicate the interpretation of data from chemical modification studies. Either the conditions necessary to perform the modification or the modified amino acids themselves could alter CT-B in such a way that refolding into a native state is no longer possible. Inability to modify a particular residue among several identical residues may prevent identification of the importance of an individual residue. Incomplete modification could lead to failure to identify specific residues as essential for biological activity. Mutational analysis avoids some of these problems and provides data that complement and extend the results of chemical modification. The study of mutants with defined substitutions of naturally occurring amino acids for specific wild-type residues facilitates interpretation of the biochemical basis for mutant phenotypes.

Several variants of CT-B have been identified that differ in two or three residues (reviewed in Finkelstein *et al.*, 1987). LTp-I and LTh-I differ in terms of their B subunits at four or five positions (Tsuji *et al.*, 1987; Leong *et al.*, 1986), and differ from CT-B at approximately 25% of the positions. All of these enterotoxins bind to GM1, but the type I LTs also bind to several glycoprotein receptors to which CT does not bind (Griffiths *et al.*, 1986). Mutant B subunits of LTp-I have been isolated that are defective in forming pentamers (Ala-64 to Val-64, Iida *et al.*, 1989; carboxyl-terminal deletions, Sandkvist *et al.*, 1990) or that are defective in binding to GM1 (glycine to aspartate-33; Tsuji *et al.*, 1985). The Asp-33 mutant of LT-B is unimpaired in its ability to form holotoxin but is non-toxic. The Gly-33 and the Ala-64 residues are both conserved in CT-B.

The role of any specific amino acid residue in the ganglioside-binding activity of CT-B can be determined, in principal, by observing which substitutions at that position are tolerated in mutant proteins without loss of function. In the present study we used site-directed mutagenesis to create substitution mutants involving Cys-9, Cys-86, Gly-33, Lys-34, Arg-35 and Trp-88. Preliminary reports involving some of these mutants were presented at the 24th and 26th Joint Conferences on Cholera and Related Diarrheal Diseases, US–Japan Co-operative Medical Science Programme (Jobling *et al.*, 1991a,b).

Results

The mutagenic oligonucleotides used in this study are listed in Table 1. Plasmids pMGJ8 and pMGJ19 were constructed as described in the *Experimental procedures*. Each plasmid contains the cloned cholera toxin (*ctx*) operon with an in-frame deletion that removes the majority of the A1 coding region but does not affect the B gene (*ctxA*$^-$, *ctxB*$^+$). Transcription of the operon from the native *V. cholerae* promoter occurs at a low level in *E. coli*. To increase the expression of wild-type or mutant *ctxB* alleles in *E. coli*, *toxR* was provided on plasmid pVM25 (Miller and Mekalanos, 1984). Mutant plasmids were numbered sequentially, beginning with pMGJ801 for plasmids

Table 1. Mutagenic oligonucleotides used to create missense mutations affecting specific residues in the B polypeptide of cholera toxin.

Oligonucleotide[a]	Sequence (5′ to 3′)[b]	Template	Strand[c] rescued
Cys-9-TCA	T T C T G C A C T C A A A T C A G T (A)	pMGJ8	–
Cys-86-AGT	G A A A A G T T A A G T G T A T G G (T)	pMGJ19	+
Gly-33-NNN	T C T C T A G C T N N N A A A A G A G A G (G G A)	pMGJ19	+
Lys-34-NNC	C T A G C T G G A N N C A G A G A G A T G (A A A)	pMGJ11	+
Arg-35-NNN	G C T G G A A A A N N N G A G A T G G C T (A G A)	pMGJ19	+
Trp-88-NNN	T T A T G T G T A N N N A A T A A T A A A A C G C C (T G G)	pMGJ19	+

a. Each oligonucleotide is named by the wild-type amino acid in mature CT-B that is affected, followed by the unique or degenerate codon used to mutate that residue in the specified template.
b. The mutant sequence is underlined and the wild-type sequence is shown beneath in parentheses. N represents an equal mixture of A, G, C or T.
c. + or – denotes the sense or antisense strand, respectively, of the CT-B gene rescued and used as the template for mutagenesis with the corresponding oligonucleotide.

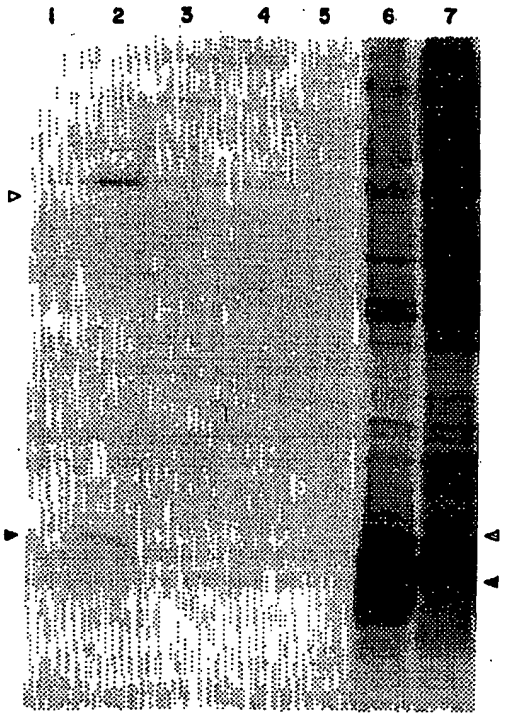

Fig. 1. [^{35}S]-methionine-labelled proteins expressed from the T7 promoter by induction of T7 RNA polymerase in *E. coli* strains bearing plasmids that code for cysteine mutants of CT-B. Lanes 1–5, periplasmic extracts; lanes 6 and 7, cell pellets. Lane 1, vector control, pKS$^-$; lanes 2 and 6, wild-type, pMGJ19; lane 3, Ser-9, pMGJ801; lane 4, Ser-86, pMGJ1901; lanes 5 and 7, Ser-9, −86, pMGJ20. Arrowheads mark approximate positions of pentameric CT-B (open), monomeric CT-B (filled), and precursor CT-B (hatched).

derived from pMGJ8, with pMGJ1901 for plasmids derived from pMGJ19, and with pMGJ1101 for derivatives of pMGJ11.

Cys-9 and Cys-86 are essential for production of mature periplasmic CT-B in E. coli

Oligonucleotide-directed site-specific mutagenesis was used to mutate each cysteine to serine. Cys-9 was mutated to Ser in pMGJ801 (TGT to AGT, using oligonucleotide Cys-9-TCA), and Cys-86 was mutated to Ser in pMGJ1901 (TGT to AGT) using the oligonucleotide Cys-86-AGT (Table 1). The Ser-9, Ser-86 double mutant, pMGJ20, was constructed by replacing an *Acc*I fragment of pMGJ1901 with the corresponding *Acc*I fragment from pMGJ801. *E. coli* DH5αF′(pVM25) carrying each of these plasmids gave no halo when tested with the radial passive immune haemolysis assay (RPIHA) for CT-B. No immunoreactive CT-B protein was detected in crude extracts of the mutant strains by the more-sensitive double antibody sandwich solid-phase radioimmunoassay (S-SPRIA). Analysis of *in vivo* labelled proteins by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE) showed that mature CT-B polypeptide (monomer or pentamer) was undetectable in periplasmic extracts of any of the cysteine mutants under conditions in which wild-type protein was detected (Fig. 1). A band corresponding to unprocessed CT-B polypeptide was detected in the pellet of polymyxin-B-extracted cells of the Ser-9, Ser-86 double mutant. The Ser-9 and Ser-86 single mutants were not tested for intracellular CT-B.

Effects of amino acid substitutions for glycine-33

Using a 64-fold degenerate oligonucleotide (Gly-33-NNN, Table 1) we generated a library of mutants of pMGJ19 potentially containing all possible substitutions for Gly-33. One hundred and ninety two individual transformants were screened for loss of receptor-binding activity by RPIHA and for base substitutions at the Gly-33 codon in colony hybridizations using an end-labelled wild-type oligonucleotide probe. The phenotypes of representative mutants in RPIH assays are shown in Fig. 2. Among 25 isolates with mutant nucleotide sequences, only three were positive by RPIHA. These three mutants all contained alternative glycine codons, strongly suggesting that only CT-B containing glycine at position 33 can produce a positive signal in RPIHA. Among the 22 RPIHA-negative mutants, 20 produced mutant CT-Bs with amino acid substitutions, and two were chain-terminating mutants. The set of 20 substitutions represented 11 of the possible 19 amino acid substitutions (Table 2). One mutant representing each substitution was selected for further study. All produced high levels of immunoreactive protein as measured by S-SPRIA (Fig. 3A). In contrast, the GM1-binding activity, as measured by GM1-SPRIA, varied greatly among the individual mutants (Fig. 3B).

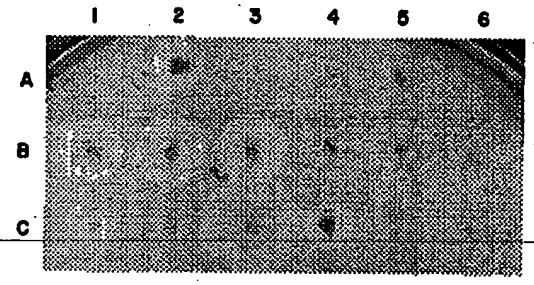

Fig. 2. RPIHA phenotype of selected mutant B subunit producing clones. *E. coli* strains carrying pVM25 and plasmids encoding various CT-B mutants were inoculated into an SRBC overlay on an LB (Ap, Cm) plate and grown overnight at 37°C. A second overlay containing Guinea-Pig Complement (BRL) and goat anti-CT serum was poured and incubated for at least 1 h at 37°C to produce visible haloes. Clones tested were: wild type (A2), Leu-33 (A3), Arg-33 (A4), Glu-33 (A5), Met-35 (B1), Tyr-35 (B2), Asn-35 (B3), Asn-88 (B4), Lys-88 (B5), Glu-88 (B6), Cys-35 (C1), Asp-35 (C2), Glu-35 (C3), wild type (C4), pKS (C5) and Glu-88 (C6).

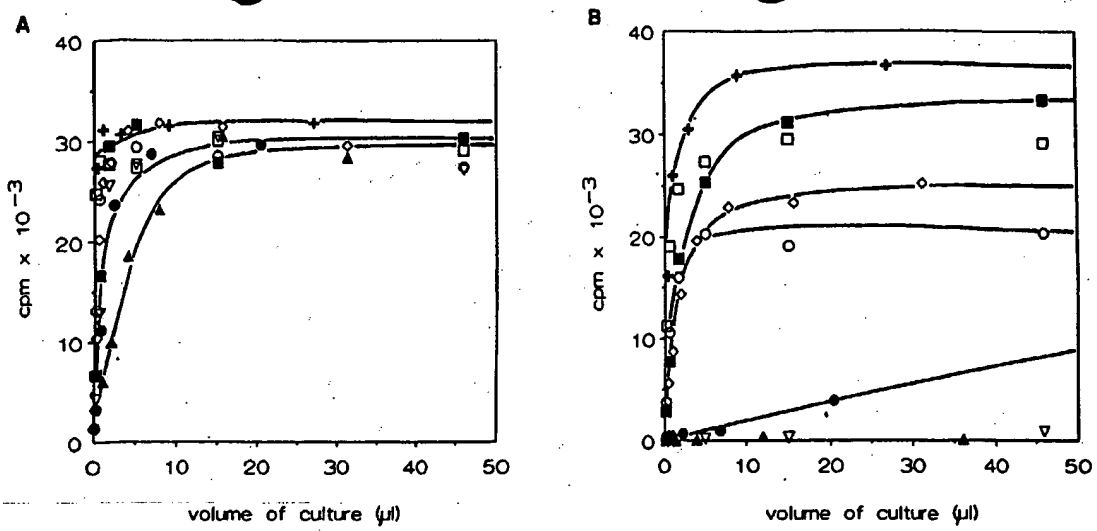

Fig. 3. Analysis of mutant CT-Bs with substitutions for Gly-33 by sandwich-SPRIA (A) and GM1-SPRIA (B). Extracts of *E. coli* strains carrying the following plasmids were tested: pMGJ19 wild type (+); pMGJ1903 Glu (▽); pMGJ1911 Ala (■); pMGJ1941 Val (●); pMGJ1943 Gln (○); pMGJ1910 Arg (□); pMGJ1949 Ile (▲); pMGJ1942 Lys (◇).

For each CT-B mutant the binding observed by S-SPRIA was directly proportional to the binding in GM1-SPRIA over an appropriate range of concentrations (Fig. 4; Olsvik *et al.*, 1983). The slopes of the lines in Fig. 4 provided quantitative measures of GM1-binding activity per unit of immunoreactive wild-type or mutant CT-B. Purified holotoxin, B pentamer or wild-type CT-B in crude extracts had a characteristic slope with an absolute value of 0.39 to 0.41. Relative binding was expressed as the slope for each mutant CT-B divided by the slope for wild-type CT-B (Table 2), and the relative binding for wild-type CT-B was assigned a value of 1. At the concentrations used for this analysis, extracts containing mutant proteins with Asp-33, Glu-33, Ile-33 or Leu-33 substitutions showed no binding to GM1. The relative binding value of the Val-33 mutant was 0.07, while Ser-33, Thr-33 and Gln-33 mutant proteins had relative binding values slightly less than that of the wild type (Table 2). Lys-33, Arg-33 and Ala-33 mutant proteins were indistinguishable from the wild type in terms of relative binding activity.

Effects of amino acid substitutions for tryptophan-88

Using the same strategy as for glycine-33 mutants, mutations at the Trp-88 codon were generated in pMGJ19, using the oligonucleotide Trp-88-NNN (Table 1). Transformants were screened by differential colony hybridization followed by RPIHA. Twenty-three mutants were identified by hybridization, and all were negative by RPIHA. Sequencing of these mutants demonstrated that 14 out of the possible 19 amino acid replacements and two termination mutants were obtained (Table 2). When

Table 2. Characterization of mutant CT-B clones.

Position substituted	MGJ no.	Amino acid	Codon	RPIHA[a] phenotype	Antigen titre[b]	Relative binding[c]
None	19	Wt[d]	Wt	+	700	1.0
Gly-33 (GGA)	1903	Glu	G A A	–	1000	0
	1950	Glu	G A G	–	ND[e]	–
	1948	Asp	G A T	–	5000	0
	1919	Ile	A T A	–	500	0.01
	1949	Ile	A T A	–	ND	–
	1954	Ile	A T T	–	ND	–
	1951	Leu	C T A	–	1250	0
	1941	Val	G T G	–	800	0.07
	1955	Val	G T A	–	ND	–
	1943	Gln	C A A	–	800	0.46
	1947	Ser	A G T	–	800	0.6
	1915	Thr	A C A	–	300	0.8

Table 2 continued.

Position substituted	MGJ no.	Amino acid	Codon	RPIHA[a] phenotype	Antigen titre[b]	Relative binding[c]
	1911 (1914)	Ala	G C G	–	2500	1.0
	1942	Lys	A A A	–	2500	1.0
	1946	Lys	A G G	–	ND	+
	1910 (1917)	Arg	C G A	–	600	1.1
	1944	Arg	A G A	–	ND	+
	1945	Arg	C G G	–	ND	+
	1916	Ochre	T A A	–	NA[f]	NA
	1918	Opal	T G A	–	NA	NA
	1934	Gly	G G C	+	ND	ND
	1935	Gly	G G A	+	ND	ND
	1940	Gly	G G G	+	ND	ND
Trp-88	1922	Ile	A T T	–	130	0.6
(TGG)	1920	Leu	T T G	–	50	0.6
	1923	Gln	C A G	–	50	0.9
	1913 (1926)	Gln	C A A	–	ND	+
	1924	Val	G T G	–	35	1.1
	1938	His	C A T	–	20	1.3
	1933	Lys	A A G	–	10	0
	1908	Lys	A A A	–	ND	–
	1936	Asn	A A T	–	4	1.1
	1907	Glu	G A A	–	3	0
	1930	Met	A T G	–	<2	+
	1927	Pro	C C A	–	<2	NA
	1904	Ser	T C T	–	<2	NA
	1906	Ser	T C G	–	<2	NA
	1931	Ser	T C A	–	<2	NA
	1912	Gly	G G A	–	<2	NA
	1937	Gly	G G G	–	<2	NA
	1921	Thr	A C C	–	<2	NA
	1926	Arg	C G A	–	<2	NA
	1902	Opal	T G A	–	NA	NA
	1925	Ochre	T A A	–	NA	NA
Arg-35	1972	Asp	G A T	–	2000	1.8
(AGA)	1971	Glu	G A A	±	2000	1.9
	1973	Cys	T G C	±	30	+
	1966	His	C A C	+	2000	1.5
	1957	Gly	G G G	+	1500	1.4
	1960	Trp	T G G	+	1200	1.4
	1961	Ser	T C A	+	3000	1.1
	1964	Tyr	T A T	+	2000	0.7
	1969 (1956)	Ile	A T A	+	2000	1.0
	1968	Met	A T G	+	>256	+
	1963	Asn	A A C	+	>256	+
	1967	Gln	C A A	+	>256	+
	1962	Arg	C G A	+	ND	ND
	1965	Arg	A G G	+	ND	ND
	1959	Amber	T A G	–	NA	NA
	1970	Opal	T G A	–	NA	NA
Lys-34	1102	Gly	G G C	±	2200	+
(AAA)	1103	Ala	G C C	±	>256	+
	1104	Ser	A G C	±	ND	+
	1105	Cys	T G C	–	10	+
	1106	Val	G T C	±	ND	+
	1109	Ile	A T C	±	7000	+
	1110	Asp	G A C	±	8000	+
	1112	Tyr	T A C	+	>256	+
	1113	Asn	A A C	+	>256	+

a. +, wild-type halo; –, no halo; ±, non-wild-type halo.
b. Dilution of extract giving 50% maximal counts by S-SPRIA.
c. Ratio of slopes of lines from GM1 binding plotted against S-SPRIA binding, for mutant divided by wild-type. + or – quantitative assays not performed but mutant CT-B bound (+) or did not bind (–) in GM1-SPRIA.
d. Wild type.
e. Not determined.
f. Not applicable.

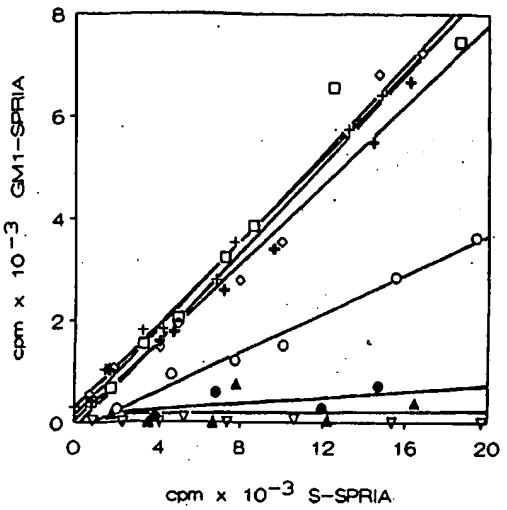

Fig. 4. Relative GM1-binding activity of selected mutant CT-Bs with substitutions for Gly-33. Each periplasmic extract was first diluted to give a signal of approximately 20000 c.p.m. by S-SPRIA, and eight additional dilutions over a 20-fold range were prepared and assayed by S-SPRIA and GM1-SPRIA. The results for the two assays for each dilution were plotted against each other. Extracts of *E. coli* TG1 carrying the following plasmids were tested: pMGJ1903 Glu (▽); pMGJ1941 Val (●); pMGJ1949 Ile (▲); pMGJ1943 Gln (○); pMGJ1910 Arg (□); pMGJ19 wild type (+). In addition, purified CT (+) and CT-B (◇) were included as controls.

tested by S-SPRIA (Fig. 5A and Table 2), none of the Trp-88 substitution mutants produced as much immunoreactive CT-B protein as the wild type. The Ile-88 mutant produced the greatest amount of immunoreactive toxin (19% of wild type, Fig. 5A). Mutants that produced lower, but still detectable, amounts of immunoreactive CT-B included Leu-88, Gln-88, Val-88, His-88, Lys-88, Asn-88, and Glu-88. Mutants that produced no detectable immunoreactive CT-B included Met-88, Pro-88, Ser-88, Gly-88, Thr-88 and Arg-88.

Although the mutant CT-Bs with substitutions for Trp-88 were produced in smaller amounts than wild-type CT-B, their relative binding to GM1 was comparable to wild-type CT-B, except for Lys-88 and Glu-88, which failed to bind (Fig. 5B, Table 2). Therefore, a positively charged residue or a negatively charged residue at position 88 abolished receptor-binding activity. The observation that the relative GM1-binding activity of mutant CT-B proteins containing Ile-88, Leu-88, Gln-88, Asn-88 or His-88 was similar to that of the wild type indicates that Trp-88 is not essential for binding of CT-B to GM1. The dramatic effect of all substitutions for Trp-88 on the amount of immunoreactive toxin suggests that Trp-88 is important for establishing or maintaining the native conformation of CT-B.

Effects of amino acid substitutions for arginine-35

Similar strategies were used to obtain and characterize substitutions for arginine-35 of *ctxB*, in pMJG19, using the oligonucleotide Arg-35-NNN (Table 1). Among 50 transformants tested, only three had mutant phenotypes by RPIHA, whereas 17 had mutant genotypes revealed by colony hybridization with the wild-type oligonucleotide probe. Most substitutions for Arg-35, therefore, do not produce mutant phenotypes by RPIHA. The three clones with no halo or with abnormal haloes were identified as Cys-35, Glu-35 and Asp-35 substitutions (Table 2). The Cys-35 mutant gave a small turbid halo and the Glu-35 mutant gave a normal-sized but turbid halo. Only the

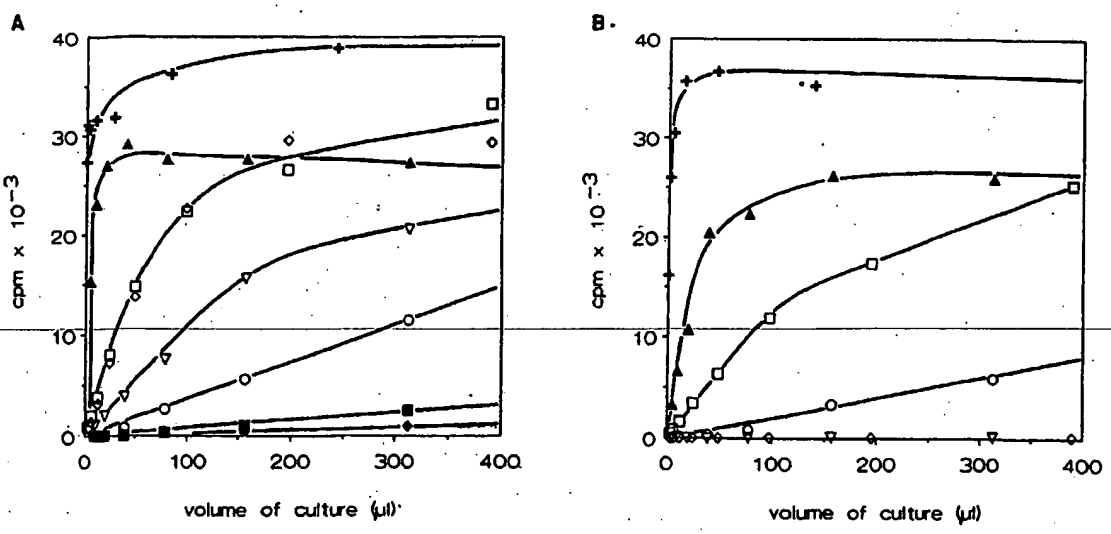

Fig. 5. Analysis of mutant CT-Bs with substitutions for Trp-88 by sandwich-SPRIA (A) and GM1-SPRIA (B). Extracts of *E. coli* strains carrying the following plasmids were tested: pMGJ19 wild type (+); pMGJ1920 Ile (▲); pMGJ1933 Lys (◇); pMGJ1938 His (□); pMGJ1907 Glu (▽); pMGJ1904 Ser (◆); pMGJ1921 Thr (■); pMGJ1936 Asn (○).

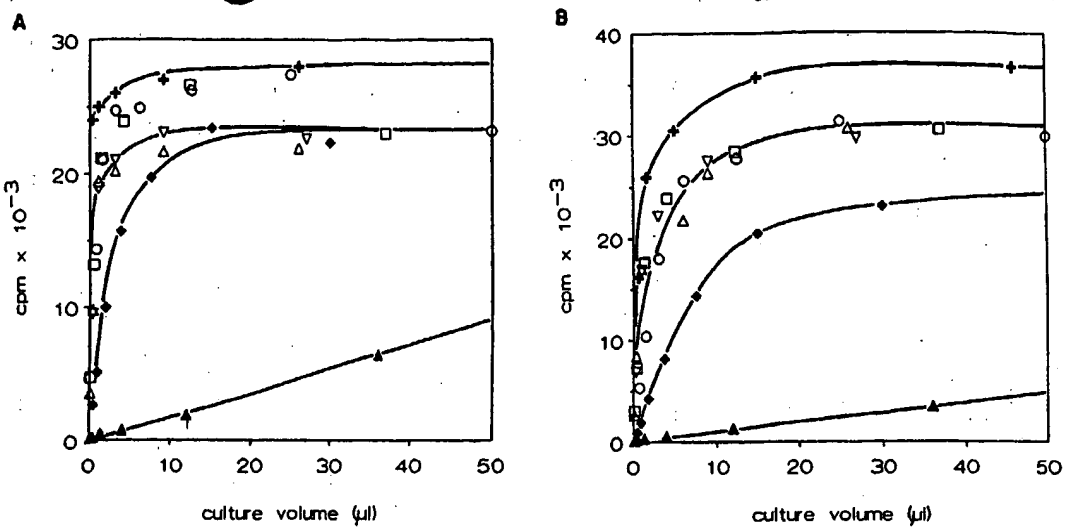

Fig. 6. Analysis of mutant CT-Bs with substitutions for Arg-35 by sandwich-SPRIA (A) and GM1-SPRIA (B). Extracts of *E. coli* strains carrying the following plasmids were tested: pMGJ19 wild type (+); pMGJ1971 Asp (△); pMGJ1972 Glu (▽); pMGJ1973 Cys (▲); pMGJ1957 Gly (○); pMGJ1966 His (□); pMGJ1960 Trp (◆).

Asp-35 mutant gave no halo. The 14 other mutants identified by hybridization included two wild-type CT-Bs with alternative arginine codons, two termination mutants, and 10 missense mutations representing nine other amino acid replacements. All of the missense mutants tested, except one, produced high levels of immunoreactive toxin that also bound to GM1 (Figs 6A, 6B). The Cys-35 mutant produced a lower level of immunoreactive CT-B. Most mutants tested, including Glu-35 and Asp-35 had relative GM1-binding values that were slightly higher than that of wild-type CT-B.

Effects of amino acid substitutions for lysine-34

Ludwig *et al.* (1985) showed that one or more lysines of the B subunit were involved in the interaction between CT-B and GM1. Because of the proximity of Lys-34 to Gly-33, we tested whether substitutions at Lys-34 would affect GM1-binding activity. Oligonucleotide Lys-34-NNC (Table 1) was designed to replace the Lys-34 codon in pMGJ11 with a codon specifying any of 15 amino acids (fixing the third position at C excluded recovery of the wild-type amino acid Arg, Met, Glu, Trp or termination codons). Twenty transformants screened by RPIHA were sequenced. Among five clones that were negative by RPIHA, two were frameshift mutations, two were IS*1* insertions at the same position within the B gene, and one was a substitution mutant (Cys-34). Eleven mutants produced normal-sized but turbid haloes, and represented six different amino acid substitutions (Gly-34, Ala-34, Ser-34, Val-34, Ile-34 and Asp-34). Two transformants with wild-type haloes, randomly selected and sequenced, had substitutions of Tyr and Asn for Lys-34. All of the mutant CT-Bs tested produced normal high levels of immunoreactive CT-B, with the exception of Cys-34. The phenotype of this mutant was similar to that of the Cys-35 mutant. All of the mutants tested bound to GM1 in the GM1-SPRIA (data not shown), but quantitative titrations were not performed for the determination of relative binding activity. The effect of negatively charged or hydrophobic amino acid substitutions for Lys-34 was very different from the effects of the corresponding substitutions for Gly-33, and Lys-34 did not appear to be involved in the interaction of CT-B with GM1.

Interaction of mutant toxins with sheep red blood cells (SRBCs)

Most of the mutant CT-B proteins retained the ability to bind to GM1 in the solid-phase immunoassay, yet failed to cause haemolysis of SRBCs in the RPIHA. As a direct test of the ability of selected mutant B subunits to bind to SRBCs, extracts were adsorbed with SRBCs and the supernatants were tested for unadsorbed CT-B (Fig. 7). All of the selected mutants with RPIHA-negative phenotypes failed to bind to SRBCs, in contrast to the extensive binding of wild-type CT-B (>80%). Previous reports demonstrated that the susceptibility of target cells to cholera toxin could be increased by incubating the cells with GM1 prior to exposing the cells to toxin (Cuatrecasas, 1973). We therefore tested whether the susceptibility of SRBCs to immune haemolysis by mutant CT-Bs could be enhanced by pretreating the SRBCs with GM1. The

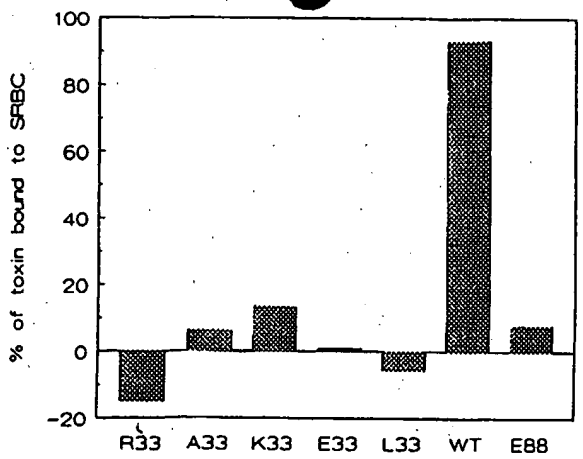

Fig. 7. Lack of binding of mutant B subunits to SRBCs. Periplasmic extracts were diluted to concentrations that gave a signal of 5000 to 15000 c.p.m. by S-SPRIA, and 100 μl aliquots were incubated with 20 μl of washed SRBCs or phosphate-buffered saline (PBS) for 1 h. SRBCs were pelleted, and the supernatants were assayed by S-SPRIA. The percentage of toxin bound was calculated as:

$$\frac{\text{(c.p.m. in PBS supernatant} - \text{c.p.m. in SRBC supernatant)} \times 100}{\text{c.p.m. in PBS supernatant}}$$

GM1-sensitized SRBCs became sensitive to immune haemolysis by the Ala-33 mutant CT-B, but remained resistant to immune haemolysis by all other mutant CT-Bs with substitutions for Gly-33.

Formation and toxicity of holotoxins containing mutant CT-Bs

To test the effect of *ctxB* mutations on holotoxin formation and activity, we introduced selected *ctxB* clones into *E. coli* carrying cloned *ctxA* and *toxR* genes on compatible plasmids. Crude extracts of these strains were tested for immunoreactivity by modified S-SPRIA and GM1-SPRIA using monoclonal anti-CT-A antibodies, and for toxicity by using the mouse Y1 adrenal cell assay (Table 3).

Most CT-B mutations, with the exceptions of Glu-35 and Asp-35, did not affect the ability of the mutant CT-Bs to associate with the A subunit to form immunoreactive holotoxin molecules, detected by the modified SPRIAs. The amounts of holotoxin antigen were proportional to the amounts of immunoreactive CT-B detected in previous assays of clones that produced only CT-B.

In most cases the relative toxicities of mutant holotoxins correlated well with the GM1-binding activities of their mutant CT-Bs. Holotoxins containing mutant CT-Bs that failed to bind to GM1 (Glu-33, Asp-33, Ile-33, Leu-33 and Val-33) were non-toxic. Most strains carrying mutant CT-Bs that bound to GM1 produced holotoxins that were of comparable toxicity to wild-type CT. The principal exceptions were the Arg-33 and Lys-33 mutants of CT-B that bound to GM1 as well as wild-type CT-B but formed holotoxins that showed no toxicity (Lys-33) or reduced toxicity (Arg-33). Holotoxin formed with Gln-33 CT-B had slightly reduced toxicity, consistent with the decreased GM1-binding activity of the Gln-33 CT-B. Only a small amount of holotoxin was formed with Ile-88 CT-B, but its relative toxicity was equivalent to that of wild-type CT. The other Trp-88 substitution mutants produced insufficient levels of CT-B to be detected in this assay. Holotoxin formed with Asp-34 mutant CT-B was produced in normal amounts and had wild-type toxicity. Holotoxins containing Glu-35 and Arg-35 mutant CT-Bs were significantly less stable than other mutant holotoxins; their toxicity and holotoxin immunoreactivity were only detectable in freshly prepared extracts, and could not be detected two days later (Table 3), although the mutant B subunits were stable and were produced in wild-type amounts.

Discussion

The production of mutant CT-Bs by oligonucleotide mutagenesis allowed us to determine the relative importance of several amino acids for the function of the B subunit of cholera toxin. Substitution of serine for Cys-9, Cys-86 or both prevented formation of the disulphide link between residues 9 and 86, and blocked formation of stable and immunoreactive CT-B. This confirms the observations of Ludwig *et al.* (1985) that reduction and carboxymethylation of these cysteine residues destroys antigenicity of CT-B, and of Hardy *et al.* (1988), who showed that reduced LT-B monomers were rapidly degraded *in vivo* in *E. coli*. This may also explain why the cysteine replacements for Lys-34 or Arg-35 were produced at low levels, since aberrant crosslinking of Cys-34 or Cys-35 to either Cys-9 or Cys-86 would cause a dramatic change in the structure of the mutant CT-B.

Substitution of aspartate for glycine at position 33 in LTp-I abolishes its ability to bind to GM1 (Tsuji *et al.*, 1985). This observation indicates that Asp-33 is incompatible with GM1-binding ability but does not establish whether or not glycine is essential. Our results with CT-B showed that several amino acids can substitute for Gly-33 without affecting GM1-binding ability. We conclude, therefore, that Gly-33 is not required for binding of CT-B to GM1. Substitution of a negatively charged or large hydrophobic residue for Gly-33 eliminated binding to GM1. Comparison of the effects of various substitutions enabled us to assess (separately) the contributions of size, charge, and hydrophobicity of the amino acid side-chain at residue 33. Among the residues with hydrophobic side-chains, Ala-33 was wild-type in its GM1-binding phenotype, but Val-33, Leu-33 and Ile-33 caused progressively decreasing GM1-binding activity. Comparison of the Glu-33 and Gln-33 mutants showed the importance of the

Table 3. Toxicity of holotoxins containing mutant CT-Bs.

Position substituted	MGJ no.	Amino acid	Toxicity (U ml^{-1})	Toxin antigen (μg ml^{-1})	Specific toxicity (U μg^{-1} antigen)	Ratio to wild type
None	19	Wt	0.80×10^3	1.50	0.53×10^3	1
Gly-33	1903	Glu	<10	8	<1.25	<0.002
	1948	Asp	<10	6	<1.67	<0.003
	1919	Ile	<10	0.25	<4.0	<0.08
	1951	Leu	<10	1.6	<6.3	<0.01
	1941	Val	<10	0.8	<12	<0.02
	1942	Lys	<10	0.5	<20	<0.04
	1946	Lys	<10	0.375	<30	<0.06
	1910	Arg	1.28×10^3	4.5	0.28×10^3	0.5
	1944	Arg	0.24×10^3	1.3	0.18×10^3	0.35
	1947	Ser	2.52×10^3	2.8	0.90×10^3	1.7
	1943	Gln	0.32×10^3	1.25	0.26×10^3	0.5
	1911	Ala	0.64×10^3	0.75	0.85×10^3	1.6
	1915	Thr	1.28×10^3	1.25	1.02×10^3	1.9
Trp-88	1922	Ile	10	0.012	0.83×10^3	1.5
Arg-35	1971	Glu	40 (<10)[a]	0.2 (0)	0.20×10^3	0.4
	1972	Asp	20 (<10)	0.06 (0)	0.33×10^3	0.6
	1960	Trp	0.20×10^3	0.44	0.45×10^3	0.85
	1964	Tyr	0.80×10^3	3.8	0.21 ± 10^3	0.4
	1963	Asn	2.60×10^3	8.0	0.32×10^3	0.6
	1966	His	0.16×10^3	0.28	0.57×10^3	1.0
Lys-34	1110	Asp	0.32×10^3	0.50	0.64×10^3	1.2

a. Values in parentheses represent assays performed 48 h after preparation of extracts.

negative charge of the carboxyl group in interfering with receptor binding. The positively charged residues arginine and lysine did not interfere with the GM1-binding activity of the mutant CT-Bs in spite of the size and length of the side-chains. We conclude that size alone is probably less important than negative charge or hydrophobicity in determining the effects of substitution for Gly-33 on GM1-binding activity of CT-B.

Substitutions at position 88, unlike those at 33, led to markedly decreased levels of immunoreactive protein detected. No amino acid substitution had a wild-type phenotype, and half of the substitution mutants made no detectable CT-B. Replacement of Trp-88 with the hydrophobic residue isoleucine, leucine or valine, or the polar residue glutamine, resulted in production of the largest amounts of immunoreactive CT-B. Other mutants produced lower amounts of mutant protein or none at all. For each amino acid substitution of Trp-88, a variable but reproducible proportion of the mutant protein was capable of being secreted and attaining an immunoreactive conformation. These mature mutant CT-Bs were as stable as wild-type CT-B (data not shown). The defect in production of these mutant CT-Bs could occur at any step in the pathway from gene to mature protein. Missense mutations are unlikely to have drastic effects on transcription or translation. The cytoplasmic CT-B mutant precursor polypeptide could be degraded prior to or during secretion across the inner membrane, or could adopt a conformation incompatible with secretion (Randall and Hardy, 1986; MacIntyre and Henning, 1990). Alternatively, the mutant polypeptides could be more susceptible to proteolysis in the periplasm or less able to fold into the wild-type conformation. The mutations appear to affect the proportion of monomers that complete these processes but do not seem to affect the stability of the immunoreactive mutant CT-B that is formed. Similar effects of substitution for a tryptophan residue of aspartate aminotransferase were noted by Mattingly, Jr, and Martinez-Carrion (1990), who showed greatly reduced yields of mutant proteins when Trp-140 was substituted with Phe or Gly. Retention of a significant level of catalytic activity by the Phe-140 mutant enzyme suggested that the importance of the Trp residue was mainly as a structural element. The low yield but near wild-type stability of the purified mutants also led to the suggestion of additional roles for the Trp residue in efficient folding and assembly of the nascent enzyme *in vivo*. All of the Trp-88 mutant CT-Bs showed near wild-type relative binding values for GM1, except for Lys-88 and Glu-88 mutants that did not bind to GM1. We conclude that Trp-88 has an important role in determining the tertiary or quaternary structure of CT-B. Although Trp-88 is not required for binding of GM1

Table 4. Plasmids, vectors and constructs.

Plasmid	Marker	Replicon	Promoter	Genotype	Source
pKS⁻	Ap	ColE1	*lac*		Stratagene
pKS⁺	Ap	ColE1	*lac*		Stratagene
M13mp19	–	M13	*lac*		Yanisch-Perron *et al.* (1985)
pACYC184	Cm Tc	p15a	*tet*		Chang and Cohen (1978)
pRK404	Tc	IncP	*lac*		Ditta *et al.* (1985)
pVM25	Cm	p15a	*tet*	*toxR*⁺	Miller and Mekalanos (1984)
pCVD30	Cm Ap	ColE1	ct	*ctxA*⁻*B*⁺	Kaper *et al.* (1986)
pCVD14	Cm Ap	ColE1	ct	*ctxA*⁺*B*⁺	Kaper *et al.* (1986)
pMGJ8	Ap	ColE1	ct	*ctxB*⁺	This study
pMGJ19	Ap	ColE1	ct	*ctxB*⁺	This study
pMGJ11	Ap	ColE1	*lac*, ct	*ctXB*⁺	This study
pMGJ14	Cm	p15a	ct	*ctxA*⁺	This study
pMGJ40	Tc	IncP	*tet*	*toxR*⁺	This study

to CT-B, its location in or near the actual receptor binding site is suggested by the effects of binding on tryptophan fluorescence (de Wolf *et al.*, 1981), the results of chemical modification studies (Ludwig *et al.*, 1985), and the phenotypes of the Lys-88 and Glu-88 mutants reported here.

All of the observed amino acid substitutions for Arg-35, except Cys-35, produced wild-type levels of immunoreactive CT-B. Most substitutions were compatible with receptor binding and binding to SRBCs by RPIHA, demonstrating that Arg-35 is not critically involved in binding to GM1. Negatively charged substitutions (Glu and Asp) did affect binding to SRBCs by RPIHA but not to purified GM1 by SPRIA. This supports the conclusions of Ludwig *et al.* (1985) that modification of any single arginine residue, specifically Arg-35, did not affect receptor binding. Our data also eliminate any requirement for Lys-34 in the GM1 binding activity of CT-B. One or more of the remaining eight lysine residues could still be involved in binding of CT-B to GM1, as proposed by Ludwig *et al.* (1985).

Residues 33, 34, and 35 of mature CT-B are able to accommodate a wide variety of amino acid substitutions without significant effects on the level of CT-B produced or its immunoreactivity, implying that these residues are located in a region of the toxin molecule that has few structural constraints on the type of side-chain present. This is in contrast with the adverse effects of most substitutions for Trp-88 on production of immunoreactive CT-B.

We noted that *in vitro* binding of mutant CT-Bs to GM1 did not correlate with binding to SRBCs. These observations suggest that the binding sites for CT-B on SRBCs that mediate complement-mediated immune haemolysis are not GM1. It is known that CT and the related LTs will bind to receptors other than GM1, although with reduced affinity (Fukuta *et al.*, 1988; Griffiths *et al.*, 1986). Because rat RBCs are known to be rich in GM1 and fucosyl-GM1 (Iwamori *et al.*, 1983) and both can function as receptors for CT, we tested them as indicator cells in the RPIHA. Results were identical to those obtained with SRBCs, in that all Gly-33 mutant extracts were unable to form a halo on either cell type. Iwamori *et al.* (1983) also detected a cryptic form of GM1 on the rat cell surface that failed to react with anti-GM1 antibodies, but CT was still able to bind to the rat erythrocytes (Iwamori *et al.*, 1985). It will be of interest to determine the nature of the active receptor for CT-B on SRBCs that mediate immune haemolysis in our RPIHAs.

Only two of the mutant CT-Bs tested (Glu-35 and Asp-35) were affected in terms of their ability to associate with CT-A to form immunoreactive CT holotoxin. Mutants with negatively charged residues at position 35 formed small amounts of unstable holotoxin. This was not because of instability of the mutant B subunit, which remained detectable over time in amounts comparable to wild-type CT-B. This finding suggests that residue 35 is located in a region of the B subunit that interacts with the A subunit in holotoxin and also that negatively charged residues at position 35 interfere with holotoxin assembly and stability.

Substitution of Gly-33 with the positively charged residue arginine or lysine did not affect the formation of holotoxin but reduced the relative toxicity of the mutant holotoxin. Decreased toxicity is not likely to be caused by decreased receptor binding, since these mutant holotoxins containing Lys-33 or Arg-33 mutant CT-Bs bound as well as wild-type CT to purified GM1 on polystyrene immunoassay plates. It is possible that these mutants are defective in a step required for toxicity that occurs after binding to the cells, e.g. a conformational change in the bound holotoxin that is believed to facilitate release of and penetration of the A subunit into and through the plasma membrane (Surewicz *et al.*, 1990).

All of the studies reported here were performed with crude extracts of mutant CT-Bs or mutant holotoxins in specific immunoassays or bioassays. The results provide important information about the roles of Cys-9, Gly-33, Lys-34, Arg-35, Cys-86 and Trp-88 for the structure and

function of CT-B. Future studies with selected purified mutant CT-Bs should provide additional information about the mechanism of the GM1 binding, assembly of CT-B pentamer and CT holotoxin, and interactions of CT with plasma membranes of specific target cells. Correlation of our observations with the three-dimensional structure of CT-B is also important but cannot be performed until the molecular structural analysis of CT or LT has been accomplished at a higher level of resolution than is currently available (Tsuji *et al.*, 1989; Spangler and Westbrook, 1989; Ribi *et al.*, 1988).

Experimental procedures

Bacterial strains and plasmids

E. coli strains TG1 (*thi, hsdD5, glnV44,* Δ(*lac-pro*), F′*traD36, proAB, lacI*^{q}, *lacZ*ΔM15; Amersham International PLC) or DH5α (*endA1, hsdR17, glnV44, thi1, recA1, gyrA, relA1,* Δ(*lacZYA–argF*)U169 Φ80d*lacZ*ΔM15; BRL) were used as the hosts for the plasmids listed below, except for the preparation of uracil-containing single-stranded (ss) DNA when CJ236 (*dut, ung, thi, relA,* pCJ105, Cm^R; BioRad) was used. Cloning vectors used are listed in Table 4.

DNA techniques and clone constructions

Mini-prep plasmid DNA was isolated using an alkaline lysis method (Morelle, 1989). Restriction and other enzymes were used as described by the manufacturers, and digestions were analysed on 0.8% agarose gels. Plasmid constructs are listed in Table 4. Plasmid pMGJ8 consists of a 1.5 kb *Hpa*II–*Hae*III fragment from pCVD30 cloned into pKS$^-$ in the opposite orientation to the *lac* promoter. Plasmid pMGJ11 consists of a 0.9 kb *Hpa*II–*Nsp*CI *ctxB*-containing fragment from pCVD30 in pKS$^-$, under the control of the *lac* promoter. Plasmid pMGJ19 consists of the same fragment in pKS$^+$ in the opposite orientation to the *lac* promoter. The *ctxA*-producing clone pMGJ14 was constructed by cloning a filled-in 1.6 kb *Acc*I fragment from pCVD14 into the filled-in *Bam*HI site of pACYC184. The *toxR*-producing clone pMGJ40 was constructed by cloning a 1.6 kb *Hin*dIII–*Bam*HI fragment from pVM25 into pRK404. DNA was transformed into cells made competent by the method of Hanahan (1983).

Oligonucleotide-directed mutagenesis and DNA sequencing

We determined the sequence of the *ctxB* gene of pCVD14, one of the two *ctx* operons from the Classical biotype *V. cholerae* strain O395 (Jobling *et al.*, 1991a) and showed it to differ at several positions from the sequence for the *ctx* operon from *V. cholerae* 2125 (Mekalanos *et al.*, 1983). Oligonucleotides were designed on the basis of our sequence; the DNA sequence coded for a protein with a predicted amino acid sequence identical to the mature CT-B peptide from *V. cholerae* 569B (Lai, 1977), as corrected by assigning aspartate instead of asparagine to amino acids 22 and 70 (Takao *et al.*, 1985). Oligonucleotides were made with an Applied Biosystems DNA synthesizer or purchased from GDI. Mutagenesis was performed as described in the BioRad *Muta-Gene Manual* but using primer to template ratios of 5:1 and an annealing temperature of 15°C (Goff *et al.*, 1987). Uracil-containing ssDNA template was prepared from CJ236(pMGJ19) using helper phage M13K07 (Pharmacia-LKB) or R408 (Russel *et al.*, 1986). The products of oligonucleotide-primed DNA synthesis reactions were transformed into *E. coli dut*$^+$, *ung*$^+$ strain TG1(pVM25), and chloramphenicol- and ampicillin-resistant (Cm^R, Ap^R) transformants were selected. Mutant colonies were detected by differential hybridization in colony blots using [^{32}P]-5′ end-labelled wild-type oligonucleotide as probe (*Amersham Mutagenesis Manual*, Amersham International PLC). Dideoxy DNA sequencing was done using *ctx*-specific primers and T7 DNA polymerase (Sequenase, USB) and reactions were analysed on buffer gradient 6 or 8% polyacrylamide–urea gels (Sheen and Seed, 1988).

Assays for CT-B and CT

Transformants were screened for production of CT-B by observing whether haloes were formed in a radial passive immune haemolysis assay (RPIHA, Bramucci and Holmes, 1978) using a sheep erythrocyte overlay on LA plates (Fig. 2). In addition, extracts were prepared from 30- to 50-fold concentrated suspensions of bacteria by treatment with polymyxin B (Finkelstein and Yang, 1983) or by sonication of 10-fold concentrated bacterial cultures. Quantification of immunoreactive CT-B was achieved using a modified sandwich solid-phase radioimmunoassay (S-SPRIA; Bramucci *et al.*, 1981) and GM1-solid-phase radioimmunoassay (GM1-SPRIA; Holmes and Twiddy, 1983). The published method for S-SPRIA was modified by substituting purified anti-CT IgG from goat serum for affinity-purified equine serum, and using monospecific rabbit anti-CT-B as the second antibody. CT holotoxin was quantified using a monoclonal anti-CT-A antibody (21B11; Holmes and Twiddy, 1983) to detect antigen bound to GM1 or goat-anti-CT-coated plates, and bound monoclonal antibody was detected by sequential incubation with rabbit anti-mouse IgG and [^{125}I]-labelled goat anti-rabbit IgG. Biological activity was determined by assessing rounding of mouse Y1 adrenal cell monolayers after exposure to toxin (Maneval *et al.*, 1980).

Protein labelling

Plasmid gene products were specifically labelled *in vivo* with [^{35}S]-methionine using the inducible T7 RNA polymerase system. One-millilitre cultures were grown and treated as described (Tabor and Richardson, 1985) and labelled with 10 μCi of [^{35}S]-methionine for 60 min. The cells were pelletted in a microfuge, resuspended in 1/10th volume of 10 mM Tris (pH 8) containing 2 mg ml^{-1} polymyxin B for 10 min at 37°C, and then pelletted. The supernatant (periplasmic extract) was mixed with an equal volume of 2× sample buffer without β-mercaptoethanol, the cell pellet was boiled in sample buffer with β-mercaptoethanol, and both were analysed by PAGE using 16%T–3%C discontinuous tricine-buffered SDS–PAGs (Schagger and Jagow, 1987). The gel was fixed, infiltrated with fluor (Jen and Thatch, 1982), and dried. Autoradiograms were made with intensifying screens using Kodak X-Omat AR film exposed at −70°C.

Media and chemicals

2YT broth (Bankier and Barrel, 1983) and LB plates (Miller, 1972) were used for routine bacterial cultures at 37°C. Antibiotics were

added where required, at the following concentrations: ampicillin, 50 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; tetracycline, 10 μg ml^{-1}.

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